Summer 8-15-2013

The Uropathogenic Escherichia coli Effector YbcL Modulates the Innate Immune Response in the Urinary Tract

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013

St. Louis, Missouri

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ACKNOWLEDGEMENTS

This work was supported by NIH grants DK076556, DK080752, and DK064540.

To the many people who supported and encouraged me along the way…

- David and My Colleagues -

- My Friends, Family and Ryan -

Thank You.

ABSTRACT OF THE DISSERTATION

The Uropathogenic *Escherichia coli* Effector YbcL Modulates the Innate Immune Response in the Urinary Tract

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Doctor of Philosophy in Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2013

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Uropathogenic *Escherichia coli* (UPEC) are the primary etiology of urinary tract infections (UTIs), one of the most common bacterial infections afflicting the human population. While UPEC cause disease throughout the urinary tract, bladder infection, or cystitis, is most prevalent. A key aspect of UPEC pathogenesis in the bladder is the modulation of the host inflammatory response. At acute time points, UPEC delay the arrival of immune cells, such as neutrophils, to the bladder. The lack of neutrophils in the bladder lumen enables UPEC to replicate freely in the urine and invade the bladder epithelium, a requirement for bacterial persistence, in the absence of immune pressure. The UPEC products responsible for delaying the arrival of immune cells to the bladder had not been identified.

This thesis work identified a bacterial protein, YbcL, that was modestly up-regulated upon UPEC exposure to either cultured bladder epithelial cells or human neutrophils. We demonstrated that YbcL suppressed the migration of neutrophils across bladder epithelia in an *in vitro* model of transuroepithelial neutrophil migration and an *in vivo* murine model of cystitis. Suppression of PMN migration by YbcL was dependent upon the presence of threonine at position 78 (T78). In fact, T78 in YbcL is highly conserved in clinical UPEC isolates,

suggesting that inhibition of neutrophil migration across epithelial barriers by YbcL is a conserved mechanism of immune modulation among UPEC.

Using a number of complementary approaches, we demonstrated that liberation of YbcL from the bacterial periplasm was required for suppression of neutrophil migration across a bladder epithelium. YbcL was detected in the supernatant and in association with bladder epithelial cells and neutrophils. Release of YbcL from the periplasm occurred in a manner that was dependent upon the concentration of YbcL in the periplasm, the duration of the infection and the presence of bladder epithelial cells. Although YbcL was soluble in the supernatant, we demonstrated that YbcL was not secreted from the periplasm by a canonical secretion system. Despite the apparent absence of a dedicated secretion system, these findings demonstrate that YbcL functions as an exoprotein.

Investigations into the mechanism underlying suppression of neutrophil migration by YbcL revealed that YbcL did not influence the production of chemoattractant molecules by bladder epithelial cells or bacteria or the ability of neutrophils to chemotax in response to stimuli, requirements for neutrophils to traverse epithelial barriers. This work identified and began the characterization of a bacterial protein, YbcL, that contributes to modulation of the innate immune response by UPEC. Additional experimentation is required to elucidate the importance of T78, the mode of delivery of YbcL from the periplasm, and the mechanism of action of YbcL. By delaying the arrival of immune cells, the activity of YbcL likely facilitates formation of the acute intracellular niche occupied by UPEC and required for persistence in the urinary tract.

CHAPTER 1

INTRODUCTION TO THE DISSERTATION

The Urinary Tract

The organs and tissues that comprise the urinary tract (i.e., the kidneys, ureters, bladder, sphincter muscles and urethra) are responsible for the production, storage and removal of waste in the form of urine from the body (1) (**Figure 1**). Urea, a component of urine, is produced when foods containing protein are broken down in the gastrointestinal tract and is carried in the bloodstream to the kidneys. The kidneys filter urea and other waste products from the blood, regulate electrolyte balance, control blood volume and maintain blood pressure through the removal of excess fluid from the bloodstream. The urine formed in the kidneys travels through two thin tubes, termed ureters, and into the bladder where it is stored. The bladder is a hollow muscular organ that expands and contracts to hold changing volumes of urine, while the sphincter muscles at the base of the bladder prevent urine from leaking out. During urination, or micturition, urine is expelled from the bladder through the urethra to the outside of the body by the coordinated relaxation of the sphincter muscles and contraction of smooth muscle in the bladder.

The primary function of the bladder is to store urine, often for long periods of time. To prevent waste products in the urine from damaging tissue or reentering the bloodstream, the bladder must be impermeable to urine contents. To that end, the epithelium that lines the bladder lumen functions as a barrier to ions, solutes and water, which can vary greatly in concentration and volume (2). This epithelium, or mucosal layer, is composed of an umbrella cell layer, an intermediate cell layer and a basal cell layer. The umbrella cells, also referred to as facet cells or

superficial cells, are highly differentiated and polarized with distinct apical and basolateral membranes separated by tight junctions (3). The luminal, or apical, plasma membranes of these cells bear hexagonal arrays of uroplakin complexes that confer membrane impermeability to urine and membrane integrity during mechanical stress (4, 5). To change the surface area of the bladder during filling and voiding, fusiform vesicles containing uroplakins are recycled to and from the plasma membrane through endocytosis and exocytosis (6-8). The urinary tract represents a highly evolved organ system that efficiently filters waste products from the blood and permits waste elimination from the body at convenient intervals.

Urinary Tract Infections

As the urinary tract is exposed to the environment, these organs are susceptible to foreign threats such as pathogenic bacteria. In fact, urinary tract infections (UTIs) are among the most common bacterial infections afflicting the human population. In the United States alone, there are more than 14 million medical visits prompted by UTI, and medical expenditures reach almost $4 billion each year (9). In addition to community-acquired infections, UTIs account for 40% of all nosocomial, or hospital-acquired, infections (10). There are 1 million cases of nosocomial UTIs in the US per year, and 80% of these can be attributed to catheterization (10, 11). UTIs have a low mortality rate. Given a high incidence in addition to a high rate of recurrence, UTIs represent a significant health burden and result in staggering health care costs.

Classifications

Bacterial pathogens access the urinary tract through the urethra which is exposed to the external environment. These organisms can replicate in the urine and colonize the bladder.

They can also ascend the ureters and colonize the kidneys. From the kidneys, bacteria can enter the bloodstream and cause sepsis. One classification scheme for UTIs is based on the infected organs. For example, infection of the bladder, or cystitis, is considered a lower UTI, while infection of the kidneys, or pyelonephritis, is termed an upper UTI. Additionally, UTIs can be described as uncomplicated or complicated. Uncomplicated UTIs typically occur in otherwise healthy individuals with no structural or functional abnormalities of the urinary tract. All other UTIs, including patients that are pregnant or have been catheterized, are considered complicated (12). These classifications can influence the choice and duration of antimicrobial therapy prescribed.

Symptomology and Diagnosis

UTIs are frequently diagnosed based on symptomology. Symptoms of cystitis include increased frequency and urgency of urination, painful urination, cloudy urine and pelvic pain. Symptoms of pyelonephritis include fever, chills, flank pain and nausea or vomiting. UTIs can also be diagnosed by culture of a clean catch urine specimen. To support a diagnosis of UTI, the urine culture must yield a known uropathogen above a certain threshold (e.g., 10^3 colony forming units (CFU) per ml of urine, although this threshold varies widely) (10, 13, 14). In addition to confirming bacteriuria, or the presence of bacteria in the urine, urine cultures also help to determine the antimicrobial susceptibility of the organism. The dipstick urinalysis, a commercially available test that detects the presence of leukocyte esterase, an enzyme released by leukocytes, and nitrites, generated by the reduction of nitrates by bacteria, represents an additional diagnostic tool (12). However, this test provides little additional information when symptoms and patient history suggest UTI. Because symptoms and bacteriuria can occur

independently and urine culture can delay diagnosis, lower UTIs are often treated based on symptomology alone.

Treatment

Lower UTIs are generally self-limiting and very rarely progress to pyelonephritis, but because of debilitating symptoms, antibiotics are usually prescribed. Antibiotic treatment of uncomplicated cystitis can range between a single dose and a ten-day course (12). Broader spectrum antimicrobial agents and longer regimens are prescribed for patients with complicated UTIs. Although antibiotics lead to faster resolution of urinary symptoms, they also have profound adverse effects on the microbiota of the gastrointestinal tract and vagina (15, 16). Antibiotics also select for resistant pathogens and commensal organisms. Despite some variation in resistance patterns, overall antibiotic resistance among pathogenic bacteria is becoming a major problem worldwide. These alarming observations are prompting physicians and researchers to reconsider standard UTI therapies. Alternative treatment and prevention options, such as the use of probiotics, adhesion inhibitors, and vaccines, are also being explored (17-19).

Recurrence

Despite effective antibiotic therapies that speed resolution of acute infection, the rate of recurrent infection is high. For example, approximately 50% of women will experience a UTI at some point in their lifetime. Of these women, 25% will experience a second UTI and 3% will experience a third in the six months after treatment of the initial UTI (20). For women who experience multiple recurrences, treatment options aside from continuous antibiotic prophylaxis are practically nonexistent. Traditionally, UTIs have been thought to be initiated by

contamination of the urinary tract with fecal flora, as the gastrointestinal tract serves as a reservoir for uropathogens and the urethral opening and rectum are in close proximity in women (20, 21). However, recent experimental evidence also suggests the existence of an intracellular reservoir formed by some uropathogens in the bladder epithelium that is resistant to antibiotic and immune clearance and is capable of initiating recurrent infection (22, 23). It is likely that both reservoirs, the gastrointestinal tract and the bladder epithelium, contribute to the high rate of recurrence. In support of these observations, about two thirds of recurrent infections in healthy women are caused by the same bacterial strain that caused the initial infection (24). Given the gastrointestinal reservoir, it is not surprising that uropathogens are thought to be transmitted directly from person to person and indirectly through contaminated food or water (10). A more thorough understanding of these bacterial reservoirs may facilitate intervention in the cycle of recurrence.

Predisposing Factors

A number of factors influence susceptibility to UTI. The female and male urinary systems are very similar except for the length of the urethra. In women, the shorter distance between the urethral opening and the bladder and the proximity of the urethral opening to the vagina and rectum, which both harbor large microbial communities, facilitates colonization of the urinary tract by pathogenic microbes. In fact, women between the ages of 15 and 30 have the highest frequency of symptomatic infection (25). Structural and functional abnormalities of the urinary tract, particularly those that affect urine flow and bladder emptying, also increase susceptibility to UTI (20, 26). In addition to anatomy, certain behaviors, such as frequent sexual intercourse and the use of spermicides, increase the likelihood of infection (27, 28). Women

with diabetes, who are pregnant, or have undergone bladder catheterization are also more prone to UTI (29-31). Previous UTIs significantly increase the likelihood of subsequent UTIs. Lastly, genetic variation in genes involved in the immune response has been correlated to increased susceptibility (32). In agreement with those findings, a history of UTIs in a first-degree female relative increases the likelihood of UTI (33). In spite of these predisposing factors, the bladder is well-equipped to prevent and clear bacterial infection.

Etiology

In addition to host factors, bacterial factors also influence whether colonization of the urinary tract results in disease or clearance. A number of bacterial species can cause UTIs, including *Klebsiella, Proteus, Staphylococcus,* and *Enterococcus* species, to name a few (10). However, the vast majority of community-acquired UTIs, greater than 80%, are caused by the gram-negative organism uropathogenic *Escherichia coli* (UPEC) (20). UPEC are a heterogeneous group of *E. coli* that are highly adapted to colonizing the urinary tract and evading clearance by immune mechanisms. These organisms encode a number of fitness and virulence factors including adhesins, capsule, fimbriae, flagella, siderophores, and toxins (34). Classified as extraintestinal pathogenic *Escherichia coli* (ExPEC), UPEC are distinct from commensal *E. coli* and from *E. coli* that cause disease in the gastrointestinal tract (35). Just as multiple *E. coli* pathotypes cause distinct gastrointestinal diseases, UPEC isolates are associated with different clinical manifestations including acute cystitis, recurrent cystitis, pyelonephritis and asymptomatic bacteriuria, or the presence of bacteria in the urine in the absence of symptoms (36). Research has focused on understanding how UPEC strains cause disease throughout the urinary tract.

Uropathogenic *Escherichia coli* Pathogenesis

As UPEC are responsible for the majority of community-acquired UTIs, researchers have dedicated significant resources to investigating the mechanisms by which UPEC survive and persist in the urinary tract. Our current understanding of UPEC pathogenesis is the result of *in vitro* experimentation, a well-characterized murine model of cystitis (37), and clinical studies analyzing samples from human patients.

Binding and Invasion

Micturition is a powerful host defense that eliminates the majority of bacteria that gain access to the lumen of the bladder. To persist in the bladder in spite of urine flow, UPEC adhere to the bladder epithelium using proteinaceous surface organelles, termed pili (**Figure 2A**) (38). Pili are composed of multiple protein subunits and are assembled by the chaperone/usher secretion pathway at the bacterial outer membrane. These highly stable fibers contain an adhesion at their tip that mediates binding to biotic and abiotic surfaces. While UPEC encode a number of different pili systems, two have been shown to be essential for colonization of the urinary tract. Type 1 pili interact with mannosylated uroplakins on the luminal surface of the bladder epithelium via the tip adhesin FimH (39). Pap, or P, pili use PapG to interact with globoseries glycolipids on the surface of kidney epithelial cells (40). These virulence organelles mediate the first, and arguably the most important, step in colonization of the urinary tract, namely adherence. UPEC strains that do not produce type 1 pili are unable to adhere to the epithelium and are eliminated from the bladder during micturition (41). It follows that small molecule inhibitors of the type 1 pilus-uroplakin interaction have been shown to treat and prevent infection in a murine model of cystitis (42, 43).

After adherence to the bladder epithelium, UPEC invade the umbrella cells and gain access to the cytoplasm (**Figure 2A**) (44). Host proteins (e.g., clathrin and dynamin) have been implicated in the invasion process (45-47). In contrast to other bacterial pathogens, UPEC invasion appears to be a passive process, as no bacterial effectors required for invasion have been identified, aside from pili. Furthermore, UPEC have been detected in fusiform vesicles, which are transported to and from the apical plasma membrane allowing changes in the surface area of the umbrella cells to accommodate changing urine volumes (48). UPEC may co-opt this cellular process to mediate their internalization, though internalized bacteria may also be expelled back into the lumen through exocytosis (49). While bacterial binding to the epithelium does not guarantee internalization, expelled bacteria may also contribute to the observation that two orders of magnitude fewer bacteria are detected intracellularly than extracellularly (50, 51). The mechanisms underlying the presumed escape of UPEC from vesicles remain unclear.

Intracellular Replication

After UPEC gain access to the cytoplasm, a phase of exponential growth begins that results in the formation of intracellular bacterial communities, or IBCs (**Figure 2B**). IBCs are large, globular masses of bacteria that have biofilm-like properties and are thought to contain between 10^5 and 10^6 organisms (52-54). IBC formation by UPEC was initially observed in a murine model of cystitis (54). In fact, multiple clinical UPEC isolates have been shown to form IBCs in multiple murine backgrounds, suggesting that IBC formation is a conserved mechanism of pathogenesis (55). Confirming the relevance of this intracellular reservoir, IBCs have been observed in urine samples from women with cystitis (56). The number of bacteria required to initiate a bladder infection in humans is unclear, though it may be relatively low. Formation of

the IBC serves to amplify the initial bacterial inoculum, and bacteria that comprise the IBC will perpetuate the infection.

The importance of IBC formation for the propagation of infection is demonstrated by the observation that UPEC strains unable to form IBCs do not persist in the murine model of cystitis. Bacterial factors involved in IBC formation have been identified, although more certainly exist. In addition to mediating binding to the bladder epithelium, type 1 pili also aid in IBC formation (51). This finding is not surprising, as surface organelles have been implicated in biofilm formation and IBCs resemble biofilms (57). In addition to type 1 pili, a UPEC strain lacking *surA* expression was also defective in IBC formation in a murine model of cystitis (50). As SurA encodes a periplasmic chaperone that aids outer membrane protein (OMP) biogenesis, it is likely that the defect in intracellular growth was due to the absence of one or more OMPs from the outer membrane. In agreement with that hypothesis, a UPEC strain lacking *ompA*, a SurA-dependent OMP, was also unable to form IBCs (58). It is unclear how the presence of OmpA promotes IBC formation. The identification of additional bacterial products that facilitate IBC formation would illuminate the cellular processes required for the development of these complex communities.

Fluxing and Filamentation

Upon maturation of the IBC, bacteria detach from the community and flux from the infected umbrella cell via cell lysis (**Figure 2C**) (53). Though some of the extracellular bacteria will be removed by micturition, other bacteria will initiate binding and invasion of neighboring naïve umbrella cells, prompting additional rounds of IBC formation and egress. A fraction of the bacteria flux from the ruptured umbrella cell as filamentous organisms. Filamentous bacteria are

more resistant to phagocytosis by immune cells than bacillary-shaped bacteria, although these organisms appear to have unique anti-phagocytosis qualities in addition to cell shape (59). The ability to form filaments is an important aspect of pathogenesis, as a UPEC mutant that was unable to filament did not form second-round IBCs (59).

Quiescent Reservoir Formation

A host defense against bacterial invasion and intracellular replication is the exfoliation of infected umbrella cells as a result of apoptosis (60-62). A toxin encoded by UPEC, α-hemolysin (HlyA), may promote this process by forming pores in bladder epithelial cell membranes (63). Umbrella cells containing IBCs have been detected in the urine of both mice and humans with cystitis (54, 56). However, the exfoliation of umbrella cells exposes the underlying intermediate cells. Bacteria present in the bladder may bind and invade intermediate cells, forming quiescent intracellular reservoirs, or QIRs (**Figure 2D**). In a murine model, UPEC in QIRs were detected in membrane-bound compartments and did not appear to exhibit growth, in stark contrast to the IBC (22). This quiescent reservoir is refractory to antibiotic and immune clearance (23). Bacteria remain viable for months in the bladder epithelium despite the absence of bacteriuria and are thought to emerge from the QIR in response to specific, though not entirely clear, signals (e.g., epithelial turnover) and initiate recurrent infection. Despite data supporting this hypothesis, direct evidence for QIRs in women is lacking. Identification of the QIR has revolutionized thinking about sources of recurrent infection. Characterization of the QIR with regard to bacterial factors required for formation and maintenance could lead to the identification of new drug targets, and disruption of QIR formation may prevent recurrent infection in some women.

Chronic and Recurrent Cystitis

Using the murine model of cystitis, researchers have demonstrated that some mouse strains (e.g., C57BL/6J) spontaneously resolve acute bladder infection, with or without the formation of QIRs, in the absence of intervention. In comparison, other murine backgrounds (e.g., C3H/HeN) are susceptible to the development of chronic cystitis, the severity of which is dependent upon the host strain and the infectious dose (64). Chronic cystitis is defined by high bacterial titers ($> 10^4$ CFU/ ml) in the urine and bladder at 4 weeks post infection (p.i.) and results in chronic inflammation, though sterilization of the bladder is not achieved. Biomarkers of chronic cystitis in mice at the local and systemic level were identified at 24 hours p.i. and included elevated levels of specific cytokines and chemokines, weight lost and severe pyuria (i.e., PMN in the urine) (64). After antibiotic therapy to resolve infection, mice that had experienced chronic cystitis were more susceptible to recurrent chronic cystitis upon inoculation with a different UPEC strain (65). These data suggest that the acute inflammatory response may predispose to chronic bacterial infection and may dictate susceptibility to recurrent cystitis. These *in vivo* findings mirror clinical observations in women who experience persistent recurrent infections despite appropriate antibiotic therapy. Additionally, these observations suggest that the magnitude of the inflammatory response must be properly regulated to achieve bacterial clearance without predisposing to chronic cystitis.

Innate Immune Response to UPEC

Mucosal tissues such as the gastrointestinal tract and the vagina harbor robust microbial communities that contribute to the overall health of the organism. In contrast, the urinary tract, aside from the urethra, has traditionally been considered a sterile mucosa. Consequently,

colonization by bacteria elicits a robust inflammatory response that is thought to cause the symptoms associated with disease. An innate immune response is initiated when pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on epithelial cells and resident immune cells. Recognition of gram-negative lipopolysaccharide (LPS) by Toll-like receptor 4 (TLR4) is an essential step in the pro-inflammatory response to UPEC in the bladder (66). In addition to LPS, TLR4 also appears to recognize type 1 and P pili (67-70). Stimulation of TLR2 and TLR5 by bacterial lipoproteins and flagellin, respectively, also contributes to the pro-inflammatory response (71, 72). Upon ligand binding, Toll-like receptors activate signaling cascades, including the NF-κB pathway, that result in changes in gene expression. Among the genes up-regulated, cytokines and chemokines such as IL-6 and IL-8 are produced and secreted (73-75). In response to the chemoattractant gradient, leukocytes, primarily polymorphonuclear leukocytes (PMN; neutrophils), migrate from the bloodstream across the epithelium and into the bladder lumen (75). Antimicrobial activities of the epithelium and innate immune cells coordinate to clear the bladder of bacteria in many cases of cystitis.

Aspects of the innate immune response in the bladder were elucidated using a murine model of cystitis and have been confirmed by human studies. Compared to the C3H/HeN background, C3H/HeJ mice had higher bacterial titers in the bladder and kidneys upon UPEC infection (76). Increased susceptibility in these mice was shown to be primarily the result of a mutation in the *Tlr4* gene (77, 78). In the absence of a TLR4 response, C3H/HeJ mice failed to recruit PMN to the bladder and, consequently, failed to clear infection (79). Analogous to the phenotype observed in C3H/HeJ mice, humans suffering from asymptomatic bacteriuria had lower TLR4 expression on their neutrophils compared to healthy controls (80). Genetic variation in *Cxcr1*, an IL-8 receptor, is correlated with increased incidence of pyelonephritis, and mice

deficient in CXCR1 are more susceptible to UTI than wild-type controls (81, 82). Lastly, IL-6, IL-8 and PMN can be detected in the urine of both mice and humans with cystitis (75, 83, 84). Murine models and human studies highlight the importance of a tightly regulated innate immune response in clearing the urinary tract of bacterial pathogens.

Immune Evasion by UPEC

UPEC are highly adapted to colonize and persist in the urinary tract, evidenced by the complex pathogenic cascade driven by UPEC in the bladder. In contrast to laboratory or commensal strains of *E. coli*, UPEC have evolved multiple, often overlapping, strategies to attenuate and subvert the innate immune response. At early time points, UPEC manipulate eukaryotic signaling cascades to delay the initiation of a pro-inflammatory response. In contrast to nonpathogenic *E. coli*, UPEC elicit significantly lower cytokine levels (e.g., IL-6 and IL-8) in culture supernatants during *in vitro* infection of cultured bladder epithelial cells (85-87). Low cytokine levels were observed even upon addition of known TLR agonists (e.g., LPS) during UPEC infection, suggesting active suppression of signaling cascades upstream of cytokine production. Genes involved in LPS biosynthesis have been implicated in this phenotype, though alterations to LPS structure appear to be only one aspect of a complex phenotype (85, 88). Klumpp and colleagues demonstrated that the clinical UPEC isolate NU14 stabilized an inhibitor of NF-κB, preventing NF-κB activation even in the presence of activating stimuli, although the mechanism underlying this phenotype is unclear (89). Cirl et al. identified a Toll/IL-1 receptor (TIR) domain-containing protein TcpC encoded by clinical isolate CFT073 that interacts with MyD88, inhibiting signaling through any pathway that requires this adaptor (90). However, TcpC homologs in clinical UPEC isolates are rare. Recently, Dhakal and Mulvey implicated α-

hemolysin (HlyA) in suppression of eukaryotic signaling during UPEC infection. HlyA indirectly mediated the degradation of many cellular proteins, including components of the NF-κB pathway (91). Corroborating these *in vitro* findings, a human study demonstrated an absence of IL-6 and IL-8 in the urine immediately before the onset of symptomatic recurrent UTI (92). These observations may be partly due to the heterogeneous nature of UPEC, but may also represent multiple complementary strategies that can be employed by a single UPEC strain to achieve the same result, namely inhibition of eukaryotic signaling pathways that initiate a pro-inflammatory response.

UPEC-mediated suppression of signaling in bladder epithelial cells delays the production of cytokines and chemokines. Compared to nonpathogenic *E. coli*, UPEC also delay the recruitment of PMN to the bladder lumen during infection (87, 93). It is unclear if this delay in PMN arrival is a consequence of inhibited chemokine production or if UPEC employ additional factors to prevent PMN from transiting the bladder epithelium. Eventually, PMN infiltrate the bladder in great numbers. In fact, PMN are readily detectable in the urine of patients with cystitis. Nevertheless, UPEC have evolved to persist in spite of a robust inflammatory response. Replication within IBCs protects UPEC from infiltrating immune cells, and filamentation allows extracellular UPEC to resist PMN engulfment. Analogous to suppression of signaling in bladder epithelial cells, UPEC also manipulate the activities of PMN. Cytotoxic necrotizing factor 1 (CNF1), a toxin secreted by UPEC, was shown to inhibit the chemotactic and phagocytic capabilities of PMN (94, 95). In addition, UPEC were shown to be less susceptible to PMN killing and elicited a less robust ROS response compared to nonpathogenic *E. coli*, though the mechanisms underlying these phenotypes are unclear (93).

In total, the activities of UPEC in the bladder promote colonization and propagation of infection. Our understanding of these processes is incomplete. It is unclear how UPEC products (e.g., LPS, TcpC, HlyA) coordinate to inhibit eukaryotic signaling, whether cascades in addition to the NF-κB pathway are blocked, and if there are additional bacterial products involved in these phenotypes. The mechanisms underlying the delayed migration of PMN to the bladder are also unclear. These initial events likely influence the outcome of infection and warrant further investigation. Suppression of the inflammatory response by UPEC is an important strategy for colonization of the bladder during acute and recurrent cystitis, but it may also facilitate asymptomatic bacteriuria and pyelonephritis.

Summary

UTIs are among the most common bacterial infections in humans. Due to the high incidence and high rate of recurrence, UTIs impose significant financial and health burdens. Because of the paucity of treatment options aside from antibiotics, treatment of UTIs is contributing to the growing global problem of antibiotic resistant organisms, which, in turn, threatens the effective treatment of UTIs. The majority of UTIs are caused by UPEC. Investigations into UPEC pathogenesis in the urinary tract may enable the identification of novel bacterial targets and the development of new anti-infective therapeutics.

During cystitis, UPEC direct a complex cascade that involves the formation of intracellular reservoirs and the manipulation of many eukaryotic processes. Intracellular replication, or IBC formation, not only allows UPEC to replicate in the presence of an abundant nutrient supply, but also serves to protect the community from infiltrating PMN and other

immune cells. Formation of IBCs is essential for the propagation of infection. Therefore, the events that precede and facilitate IBC formation are important steps in the pathogenic cascade.

At early time points, UPEC delay the arrival of PMN to the bladder, allowing invasion of the bladder epithelium and establishment of the intracellular niche in the absence of immune pressure. In other words, the delayed arrival of PMN promotes IBC formation. It would stand to reason that accelerated PMN arrival to the bladder would limit bacterial invasion into the epithelium through bacterial killing and, consequently, disrupt formation of the intracellular reservoir. Interference in UPEC-mediated suppression of PMN migration may aid in bacterial clearance.

UPEC inhibit the production of cytokines and chemokines by the bladder epithelium at early time points, and some of the bacterial products involved in this phenotype have been identified. It is unclear if delayed PMN recruitment is due to the absence of epithelial-derived chemokines. Given the overlapping functions of previously identified bacterial products, it is likely that UPEC encode additional factors that specifically mediate inhibition of PMN migration. Such proteins have not been identified thus far. Suppression of the acute inflammatory response by UPEC represents an important step in the pathogenic cascade that could be manipulated via therapeutic intervention, and consequently, deserves investigation.

Tables and Figures

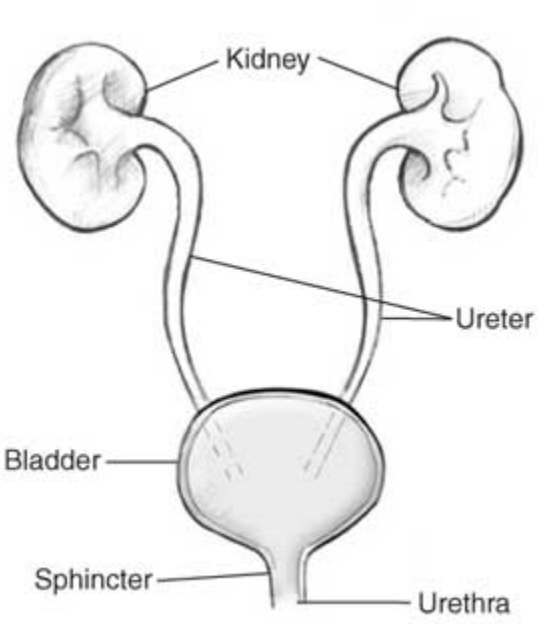

Figure 1: Schematic of the Urinary Tract System. The urinary tract is tasked with producing, storing and eliminating waste in the form of urine from the body. The urinary tract is composed of two kidneys, two ureters, the bladder, sphincter muscles and urethra. Urine is produced in the kidneys and is expelled from the body through the urethra. Adapted from the National Kidney and Urologic Diseases Information Clearinghouse (NKUDIC) (1).

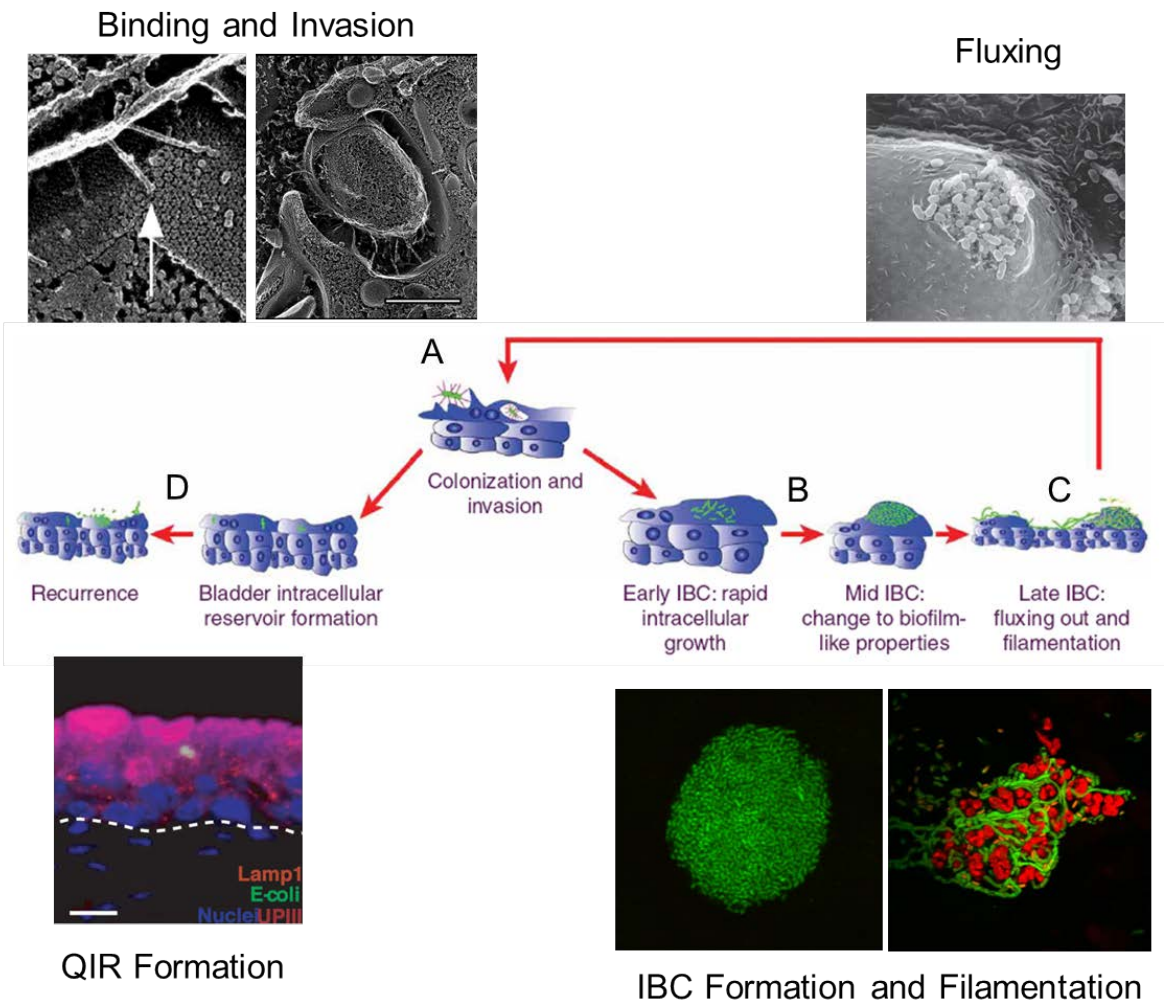

Figure 2: UPEC Infectious Cycle in the Bladder. (A) UPEC bind and invade umbrella cells of the bladder epithelium in a type I pili-dependent manner. (B) In the cell cytoplasm, UPEC rapidly grow and divide forming large collections of bacteria, termed intracellular bacterial communities (IBCs). (C) Eventually, UPEC detach from the IBC and flux from the infected cell, in either a filamentous or bacillary morphology. Extracellular bacteria initiate subsequent rounds of IBC formation by invading neighboring umbrella cells. (D) In addition to the acute reservoir, UPEC also form a quiescent intracellular reservoir (QIR) that is thought to seed reinfection. In addition to chronic persistence via QIRs, UPEC can also cause chronic active cystitis in some murine backgrounds.

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CHAPTER TWO

YBCL OF UROPATHOGENIC *ESCHERICHIA COLI* SUPPRESSES TRANSEPITHELIAL NEUTROPHIL MIGRATION

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Modified from Lau et al. (2012) *Infect Immun*.

Abstract

Uropathogenic *E. coli* (UPEC) 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 Δ*ybcL*) failed to suppress transepithelial 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 ($YbcL_{MG}$) failed to complement the UTI89 Δ*ybcL* defect, whereas expression of the UPEC YbcL variant ($YbcL_{UTI}$) 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 $YbcL_{UTI}$ protein suppressed PMN migration in response to live or killed MG1655, and $YbcL_{UTI}$ was detected in the supernatant during UPEC infection of bladder epithelial cells or PMN. Lastly, early PMN influx to murine bladder tissue was augmented upon in vivo infection with UTI89 Δ*ybcL* compared with wild-type UPEC. Our findings demonstrate a role for UPEC YbcL in suppression of the innate immune response during urinary tract infection.

Introduction

Urinary tract infections (UTI) are among the most common bacterial infections in the United States, resulting in over $2 billion in direct and indirect costs (11). Uncomplicated UTI primarily afflict otherwise healthy women, though anatomical and urodynamic abnormalities, 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 six 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 reinoculation 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) that employ a variety of strategies to effectively colonize and persist within the urinary tract. This is evidenced by an array of disease manifestations that 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 have acquired mechanisms to modulate the innate immune response during acute infection to access the intracellular niche (reviewed in (17)). Recent studies have demonstrated inhibition of pro-inflammatory 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 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 (5, 6, 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* encode YbcL homologs, only the uropathogenic variant, $YbcL_{UTI}$, 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 conferred upon the nonpathogenic strain of *E. coli* K-12 MG1655 by episomal expression of the $YbcL_{UTI}$ variant or by addition of purified $YbcL_{UTI}$ protein to the bacterial inoculum. Furthermore, $YbcL_{UTI}$ was detected in the supernatant during UPEC infection of bladder epithelial cells and PMN in vitro, and $YbcL_{UTI}$ suppressed PMN migration 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 β-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 30-min incubation at 55°C, and an aliquot of the suspension was plated to confirm bacterial death. UTI89 *ybcL::cat* (also denoted UTI89 Δ*ybcL*) was created by linear transformation of UTI89/pKM208 (27) with a fragment amplified from template plasmid pKD3 (8) using the primers JLP266 and JLP267 (primer sequences are given in **Table 1**); the deletion was verified by direct sequencing. For complementation experiments, a plasmid encoding YbcL with a C-terminal FLAG tag under the control of an IPTG-inducible promoter was constructed. The *ybcL* open reading frame (ORF) encoded by UTI89 was amplified from genomic DNA using primers MEL23 and MEL24, with the reverse primer containing the FLAG epitope sequence. The fragment was digested with BamHI and XbaI and then ligated into pTRC99A (Amp^r) which had been similarly digested. Transformed clones of *E. coli* Top10 (Invitrogen) were selected on ampicillin plates and tested by colony PCR. Accuracy of the resulting $pYbcL_{UTI}$ construct was confirmed by direct sequencing. Using a similar strategy, *ybcL* encoded by MG1655 was amplified using primers MEL62 and MEL24 and ligated into pTRC99A to generate $pYbcL_{MG}$. The QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to generate point mutations at the *ybcL* codon for residue 78. Primers MEL69 and MEL70 and template plasmid $pYbcL_{UTI}$ were used to generate $pYbcL_{UTI(T78V)}$. Primers MEL237 and MEL238 and template plasmid $pYbcL_{UTI}$ were used to

generate pYbcL$_{UTI(T78A)}$. Primers MEL67 and MEL68 and template plasmid pYbcL$_{MG}$ were used to generate pYbcL$_{MG(V78T)}$. The expected mutations were verified by direct sequencing.

Membrane-tethered YbcL variants were designed according to the findings of Yamaguchi and colleagues (34). To tether YbcL to the bacterial inner membrane (YbcL$_{IM}$), we generated a fusion protein between NlpA (an inner membrane lipoprotein) and the mature form of YbcL. Using UTI89 genomic DNA as template, code for the signal sequence and the first 12 amino acids of NlpA as well as a region homologous to the N-terminus of YbcL was amplified by primers MEL286 and MEL287. Sequence encoding the mature form of YbcL including a region homologous to NlpA was amplified by primers MEL288 and MEL24. These PCR products were annealed and extended by PCR, digested with BamHI and XbaI and ligated into pTRC99A. Using a similar approach, we generated an NlpA-YbcL fusion protein that localized to the outer membrane (YbcL$_{OM}$) by mutating the second amino acid in NlpA from an aspartic acid to a serine (34). Primers MEL286 and MEL289 were used to amplify the NlpA product containing the amino acid mutation, and primers MEL290 and MEL24 were used to amplify the YbcL product. The PCR products were cloned into pTRC99A as described above. The two constructs were verified by direct sequencing. Equivalent expression of all YbcL variants after IPTG induction was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of bacterial fractions.

Tissue culture.

The 5637 bladder epithelial cell line (derived from bladder carcinoma; ATCC HTB-9) was obtained from the American Type Culture Collection. Cells were cultured and experiments were conducted in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Sigma)

at 37°C in a humidified atmosphere with 5% CO_2 unless otherwise noted. Preparation of inverted epithelial monolayers has been previously described (23). Briefly, ~ 10^5 5637 cells were seeded on an inverted Transwell insert (0.33cm^2 polycarbonate membranes with 3-µm diameter pores, Corning #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, 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 10^7 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 PBS and diluted in RPMI. A bacterial inoculum of 6×10^6 CFU/ml (a multiplicity of infection (MOI) of 40 CFU/cell) or equivalent volume of RPMI was applied to the apical side 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 #3473) containing 0.6 ml RPMI and 10^6 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 of at least three independent experiments. Statistically significant differences were determined using an unpaired Student's *t* test.

To generate conditioned media, 5637 cells grown to confluence in 15-cm dishes were infected with the indicated strains of *E. coli* at a MOI 40. After 1 h incubation at 37°C, the supernatant was collected and filter sterilized using syringe-driven filter units (0.22μm pore size, Millipore). The filter-sterilized supernatant (conditioned media) 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 media was assessed as described above.

YbcL localization by Western blot.

To mimic the transepithelial PMN migration assay, 5637 cells or freshly isolated PMN were infected with the indicated strains of *E. coli* at a MOI of 40 or 10, respectively. After 1 h 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 (0.22μm pore size, Millipore), and protein was precipitated using 15% trichloroacetic acid (TCA) (Sigma). Samples

were separated by SDS-PAGE using 4-20% precast gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 2% nonfat milk + 2% bovine serum albumin (BSA) (Sigma), blots were probed with mouse anti-FLAG antibody (1:1000, Sigma) followed by goat anti-mouse IgG antibody (1:2000, Sigma) and were developed using Tropix CDP-Star (Applied Biosystems).

Sequencing 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 Dr. 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 Pfu 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 prevalence of specific amino acids at position 78 in YbcL from various *E. coli* groups was compared using Fisher's exact test.

Purification of YbcL variants.

ybcL alleles were amplified from the constructs described above using the following primer sets: $pYbcL_{UTI}$ and $pYbcL_{UTI(T78V),}$ MEL23 and MEL30; and $pYbcL_{MG}$ and $pYbcL_{MG(V78T),}$ MEL62 and MEL30, where the reverse primer contains a sequence encoding a 6-histidine tag (6xHis) in

place of the FLAG epitope. The amplicons 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. Periplasms were prepared from *E. coli* Top10 carrying these plasmids and dialyzed overnight in PBS before being applied to a Ni-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 bicinchoninic acid protein assay (Thermo Scientific).

Murine cystitis and tissue myeloperoxidase 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 (2.5×10^7 CFU) or sterile PBS. At 1 h post infection (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 mean 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) and J. Loughman, unpublished data), we identified a periplasmic protein YbcL with structural homology to mammalian Raf-1 kinase inhibitory protein (RKIP) (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 uroepithelial 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* strain MG1655 stimulated robust PMN migration, while infection with the UPEC strain UTI89 resulted in significantly fewer PMN in the lower reservoir (**Figure 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 co-infection with MG1655 plus UTI89 yields the uropathogenic phenotype (23). In contrast to wild-type UTI89, UTI89 Δ*ybcL* elicited significantly more PMN ($p < 0.0001$), and episomal expression of YbcL in the *ybcL* mutant restored wild-type levels of PMN migration (**Figure 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 YbcL$_{UTI}$ 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 semi-conservative changes (**Figure 2A and B,** blue). In the single non-conservative difference, the UTI89 variant (denoted YbcL$_{UTI}$) contains a threonine at position 78, while the MG1655 variant (YbcL$_{MG}$) contains a valine (**Figure 2A and B,** green). The crystal structure of YbcL encoded by K-12 strain W3110 (100% identical at the amino acid level to YbcL$_{MG}$) has been solved (32). However, any effect these amino acid differences may have on the tertiary structure of YbcL$_{UTI}$ 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 YbcL$_{MG}$ variant failed to complement UTI89 Δ*ybcL* in the transepithelial PMN migration model (**Figure 2C;** $p < 0.0001$ compared to UTI89). To assess the importance of the non-conservative amino acid substitution in suppression of PMN migration

by $YbcL_{UTI}$, we generated additional YbcL variants. Expression of $YbcL_{UTI(T78V)}$ (containing a threonine-to-valine mutation at position 78) in UTI89 Δ*ybcL* did not suppress PMN migration (**Figure 2C;** $p < 0.005$), demonstrating that this mutation resulted in a loss of function for the uropathogenic variant. Conversely, expression of $YbcL_{MG(V78T)}$ (containing a valine-to-threonine mutation at position 78) in UTI89 Δ*ybcL* reduced PMN levels in the lower reservoir (**Figure 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* and highlight the importance of threonine 78 in YbcL for UPEC-mediated suppression of PMN migration.

Given the functional consequence of the non-conservative 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), enteroaggregative *E. coli* (EAEC) 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 compared to 39% of uncharacterized fecal isolates, 14% of GIPEC (all that contained T78 were AIEC), and 0% of laboratory strains (**Figure 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 (**Figure 2E**). In total, 83% of UPEC (including both sequenced and clinical strains) compared to 25% of other *E. coli* 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 $YbcL_{UTI(T78A)}$ failed to complement UTI89 Δ*ybcL* in the transepithelial PMN migration model (**Figure 2C;** $p < 0.0001$). Taken together, these data demonstrate the prevalence of threonine 78 in YbcL among UPEC and illustrate its importance in suppression of the innate immune response by these diverse uropathogens.

$YbcL_{UTI}$ confers suppressive activity on nonpathogenic E. coli MG1655.

We next aimed to investigate whether other UPEC-encoded factors were required for $YbcL_{UTI}$-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 $YbcL_{UTI}$ or $YbcL_{MG(V78T)}$ in MG1655 yielded PMN migration levels similar to wild-type UTI89 (**Figure 3A**), demonstrating conferral of the uropathogenic phenotype upon the nonpathogenic strain. In contrast, episomal expression of $YbcL_{MG}$ or $YbcL_{UTI(T78V)}$ in MG1655 allowed significantly more PMN migration than UTI89 (**Figure 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 $pYbcL_{UTI}$, as determined by Western blot (data not shown). The addition of purified $YbcL_{UTI}$ or $YbcL_{MG(V78T)}$ upon infection with MG1655 resulted in PMN levels similar to infection with UTI89 (**Figure 3B**). Conversely, MG1655 plus purified $YbcL_{MG}$ or $YbcL_{UTI(T78V)}$ stimulated significantly more PMN migration than the uropathogen UTI89 (**Figure 3B;** $p < 0.01$). Analogous experiments conducted using these purified YbcL variants and UTI89 Δ*ybcL* as the bacterial stimulus resulted in the same trends in PMN migration (data not shown). Furthermore, $YbcL_{UTI}$ 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 (**Figure 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 (**Figure 3D;** $p < 0.05$). Infection with HKMG plus purified $YbcL_{UTI}$ or $YbcL_{MG(V78T)}$ yielded the uropathogenic phenotype, eliciting low levels of PMN migration similar to UTI89 (**Figure 3D**), while the addition of $YbcL_{MG}$ or $YbcL_{UTI(T78V)}$ to the same bacterial stimulus had no effect on PMN migration (**Figure 3D;** $p < 0.005$), in agreement with data generated using live MG1655. Taken together, these data demonstrate that $YbcL_{UTI}$ 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 for 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 Δ*ybcL* in the transepithelial PMN migration model. UTI89 Δ*ybcL* episomally expressing either $YbcL_{IM}$ or $YbcL_{OM}$ stimulated significantly more PMN migration than wild-type UTI89 (**Figure 4A**; $p \leq 0.005$). 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 a MOI 40 for 1 h at 37°C. The supernatant (conditioned media) was filter sterilized and used in place of the bacterial inoculum in the transepithelial PMN migration model. Conditioned media from infection of 5637 cells with UTI89 stimulated a low level of PMN migration (**Figure 4B**). In contrast, conditioned media generated during UTI89 Δ*ybcL* infection stimulated significantly more PMN migration (**Figure 4B**; $p < 0.01$). This phenotype could be reversed by expression of $YbcL_{UTI}$ in UTI89 Δ*ybcL*, but not 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 a 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, $YbcL_{UTI}$ and $YbcL_{OM}$ were clearly detected in the supernatant, in contrast to $YbcL_{IM}$ which was minimally detected in that fraction (**Figure 4C**). All three YbcL variants were detected in the PMN lysate. However, only $YbcL_{UTI}$ was detected in the 5637 cell lysate (**Figure 4C**). When these cell types were infected with either MG1655 or UTI89 Δ*ybcL* episomally expressing the MG1655 YbcL variant, $YbcL_{MG}$ exhibited the same localization pattern as $YbcL_{UTI}$ (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 $YbcL_{UTI}$ is secreted from the bacterial periplasm during infection of bladder epithelial cells and PMN. Although it was detected in the supernatant, $YbcL_{OM}$ did not complement the *ybcL* mutant in the transepithelial PMN migration model, suggesting that localization to the supernatant is not sufficient for suppression of PMN migration by $YbcL_{UTI}$.

$YbcL_{UTI}$ suppresses acute PMN migration in vivo.

We used a murine model of cystitis to assess a potential contribution by $YbcL_{UTI}$ 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 Δ*ybcL* elicited significantly more PMN than wild-type UTI89 (**Figure 5A;** $p < 0.0001$). Suppression of PMN migration was nearly completely restored to the *ybcL* mutant upon complementation with pYbcL$_{UTI}$ (**Figure 5A**). Modestly lower bacterial titers were recovered after infection with the *ybcL* mutant or the complemented strain compared to wild-type UTI89 (**Figure 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 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 Δ*ybcL* formed IBCs that were indistinguishable from wild-type UTI89 as assessed by confocal fluorescent microscopy (data not shown), and bacterial titers recovered from wild-type or *ybcL* mutant-infected mice were similar at 6, 16, 24, 48, hours p.i., 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 semi-conservative and one non-conservative amino acid difference compared to the UTI89 homolog. We demonstrated that threonine at the non-conservative position 78 is required for suppression of PMN migration by the uropathogenic

variant $YbcL_{UTI}$. In contrast, the nonpathogenic variant $YbcL_{MG}$ 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 investigate 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 UPEC in addition to the resident (commensal) microflora and supports a considerable amount of horizontal gene transfer. Like UPEC in the urinary tract, GIPEC influence local immune responses within the GI tract (7), although it is unclear if YbcL homologs contribute to this phenotype in the gut. Given that the majority of GIPEC encode valine- or alanine-containing YbcL homologs, immune modulation in the GI tract by these strains more likely occurs independently of YbcL. In contrast, the prevalence of threonine 78 among UPEC-encoded YbcL homologs suggests that suppression of PMN migration by YbcL is a conserved mechanism of innate immune modulation within the urinary tract. As threonine-containing YbcL homologs are present in UPEC strains that cause asymptomatic bacteriuria and pyelonephritis in addition to acute and recurrent cystitis, it is likely that YbcL contributes to pathogenesis throughout the urinary tract.

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 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 $YbcL_{UTI}$, 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 Δ*ybcL*-infected mice, suggesting that YbcL facilitates the establishment of UTI rather than persistence. Considering the large bacterial inoculum (~10^7 CFU) and the capacity of IBCs to amplify and propagate infection, it is not surprising that increased PMN recruitment in the UTI89 Δ*ybcL*-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 $YbcL_{UTI}$ may significantly favor bacterial survival prior to epithelial invasion, tipping the balance toward infection rather than clearance.

Suppression of PMN migration by $YbcL_{UTI}$ was conferred by episomal expression or the addition of purified protein to either live or nonviable MG1655, demonstrating that $YbcL_{UTI}$ 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 $YbcL_{UTI}$ by Western blot in filter-sterilized, TCA-precipitated conditioned media from UTI89/p$YbcL_{UTI}$ Δ*ybcL* grown in LB (M. Lau and D. Hunstad, unpublished data), suggesting that secretion of $YbcL_{UTI}$ is regulated. Given that the localization pattern of $YbcL_{MG}$ mimicked the pattern of $YbcL_{UTI}$ 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 pathogen-specific as the localization pattern of the YbcL variants ($YbcL_{UTI}$ and $YbcL_{MG}$) was independent of the bacterial strain, MG1655 or UTI89 Δ*ybcL*. In light of these observations, we hypothesize that secretion of $YbcL_{UTI}$, 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 $YbcL_{OM}$ in the supernatant fraction during UPEC infection is associated with OMVs and 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 $YbcL_{UTI}$ 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 $YbcL_{IM}$ 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 addition to the supernatant and PMN lysate, $YbcL_{UTI}$ 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 $YbcL_{UTI}$. 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 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 in vitro 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 $YbcL_{UTI}$ (M. Lau and D. Hunstad, unpublished data), demonstrating that $YbcL_{UTI}$ and RKIP have distinct functions despite their structural homology. Furthermore, UTI89 Δ*ybcL*, like wild-type UTI89, elicits minimal IL-6 and IL-8 from cultured bladder epithelial cells or human PMN relative to MG1655 (M. Lau and D. 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 $YbcL_{UTI}$ required to suppress PMN migration, and the presence of $YbcL_{UTI}$ in eukaryotic cell lysates, we hypothesize that $YbcL_{UTI}$ inhibits a eukaryotic signaling cascade that promotes transepithelial PMN migration. Ongoing work aims to elucidate the mechanism underlying the differential PMN migration and the role that $YbcL_{UTI}$ 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.

Acknowledgements

This work was supported by National Institutes of Health (NIH) grants R01-DK080752 and R03-DK076556 (to D.A.H.). Clinical isolates were collected under NIH grant P50-DK064540. We thank K. Tiemann for technical assistance, and D. Haslam and D. Berg for critical review of the manuscript.

Tables and Figures

Table 1: Primers used in this study

Primer	Sequence (5’ → 3’)
JLP266	TCGTTTCAAGTGTATTGGCATTCATAACATTTTCTGCGCAGTGTAGGCTG GAGCTGCTTC
JLP267	TAAACTGGTGTTATCTCAGCGGTTGCAATTTTATTGGCATCATATGAATA TCCTCCTTAG
MEL23	GCATGGATCCGGTCACAACAATGAGG
MEL24	GCATTCTAGACTACTTGTCATCGTCGTCCTTGTAGTCCTTTATCTCATAAA CT
MEL30	GCATTCTAGACTAGTGGTGATGGTGATGATGCTTTATCTCATAAACT
MEL62	GCATGGATCCGGTCATAACAAAGAGGT
MEL67	GCAACAGTAACATATTTGCCCACTGATGCAGGGAGACGTGATGG
MEL68	CCATCACGTCTCCCTGCATCAGTGGGCAAATATGTTACTGTTGC
MEL69	GCAACTGTAACATATTTGCCCGTTGATGCAGGAAGACGTGATGG
MEL70	CCATCACGTCTTCCTGCATCAACGGGCAAATATGTTACAGTTGC
MEL231	ATGAAAAMACTTATCGTTTCAA
MEL232	CTACTTTATCTCATAAACTGGTG
MEL237	GCAACTGTAACATATTTGCCCGCTGATGCAGGAAGACGTGATGG
MEL238	CCATCACGTCTTCCTGCATCAGCGGGCAAATATGTTACAGTTGC
MEL286	GCAT GGATCC ATGAAACTGACAACACATCATCTACGGGCG
MEL287	GTTGCTCTCCTGTTTTTATTTCATTACTAGTGACCTGAAATTTAATATGCT TTTCATCGC
MEL288	AGGTTGCGACCAGAGCAGCAGCGATGAAAAGCATATTAAATTTCAGGTC ACTAGTAATGA
MEL289	TTCATTACTAGTGACCTGAAATTTAATATGCTTTTCATCGCTGCTGCTCTG GCTGCAACC
MEL290	AGGTTGCAGCCAGAGCAGCAGCGATGAAAAGCATATTAAATTTCAGGTC ACTAGTAATGA

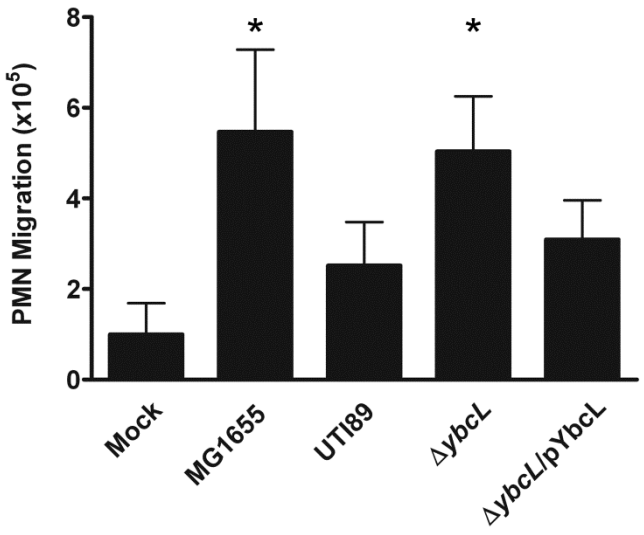

Figure 1: UPEC YbcL suppresses transepithelial PMN migration *in vitro*. 5637 bladder epithelial cell monolayers grown on Transwell inserts were infected at their apical surfaces with the indicated strains of *E. coli* or mock infected, and freshly isolated human PMN were added at the basolateral surface.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

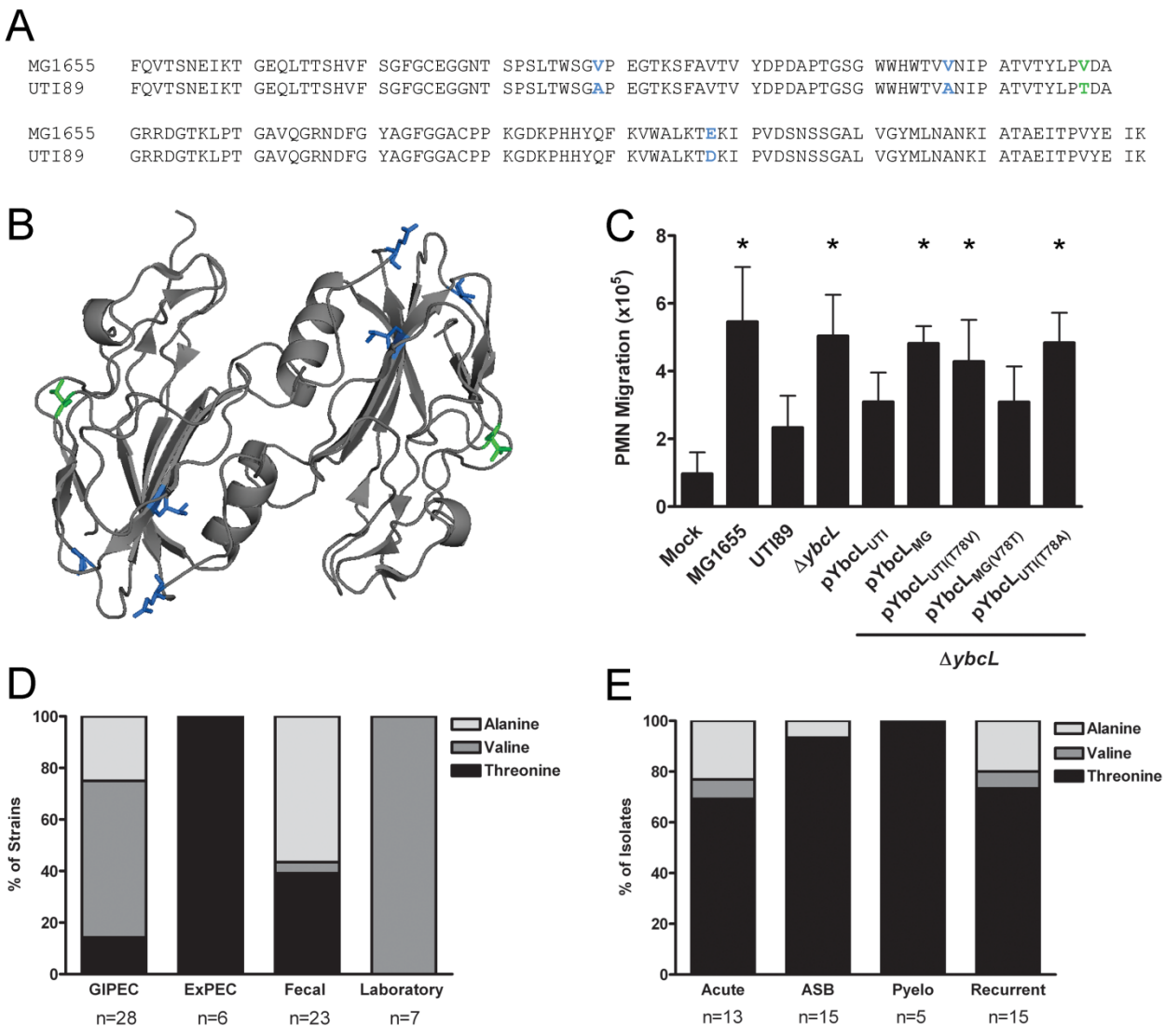

Figure 2: Threonine 78 is required for suppression of PMN migration by $YbcL_{UTI}$. Amino acid alignment of mature YbcL homologs encoded by MG1655 and UTI89 (A) and a ribbon diagram of YbcL encoded by K-12 strain W3110 (dimeric as in its crystal structure (32)) (B) are shown. Conservative and semi-conservative differences are depicted in blue, and the non-conservative difference is depicted in green. (C) Complementation of UTI89 Δ*ybcL* by episomal expression of YbcL variants was assessed using an in vitro model of transepithelial PMN migration. Experiments were conducted and data are represented as described in Figure 1. An

asterisk denotes a statistically significant ($p < 0.005$) increase in PMN migration compared to wild-type UTI89. The presence of threonine at position 78 correlates with the suppression of PMN migration by YbcL. The distribution of amino acids at position 78 in YbcL homologs encoded by sequenced *E. coli* strains (D) or clinical UPEC isolates ((E); asymptomatic bacteriuria (ASB), acute cystitis (Acute), recurrent cystitis (Recurrent) and pyelonephritis (Pyelo)) is shown. Overall, threonine 78 is present in 83% of UPEC strains (43/52) compared to 25% of other *E. coli* strains (15/60) ($p < 0.0001$).

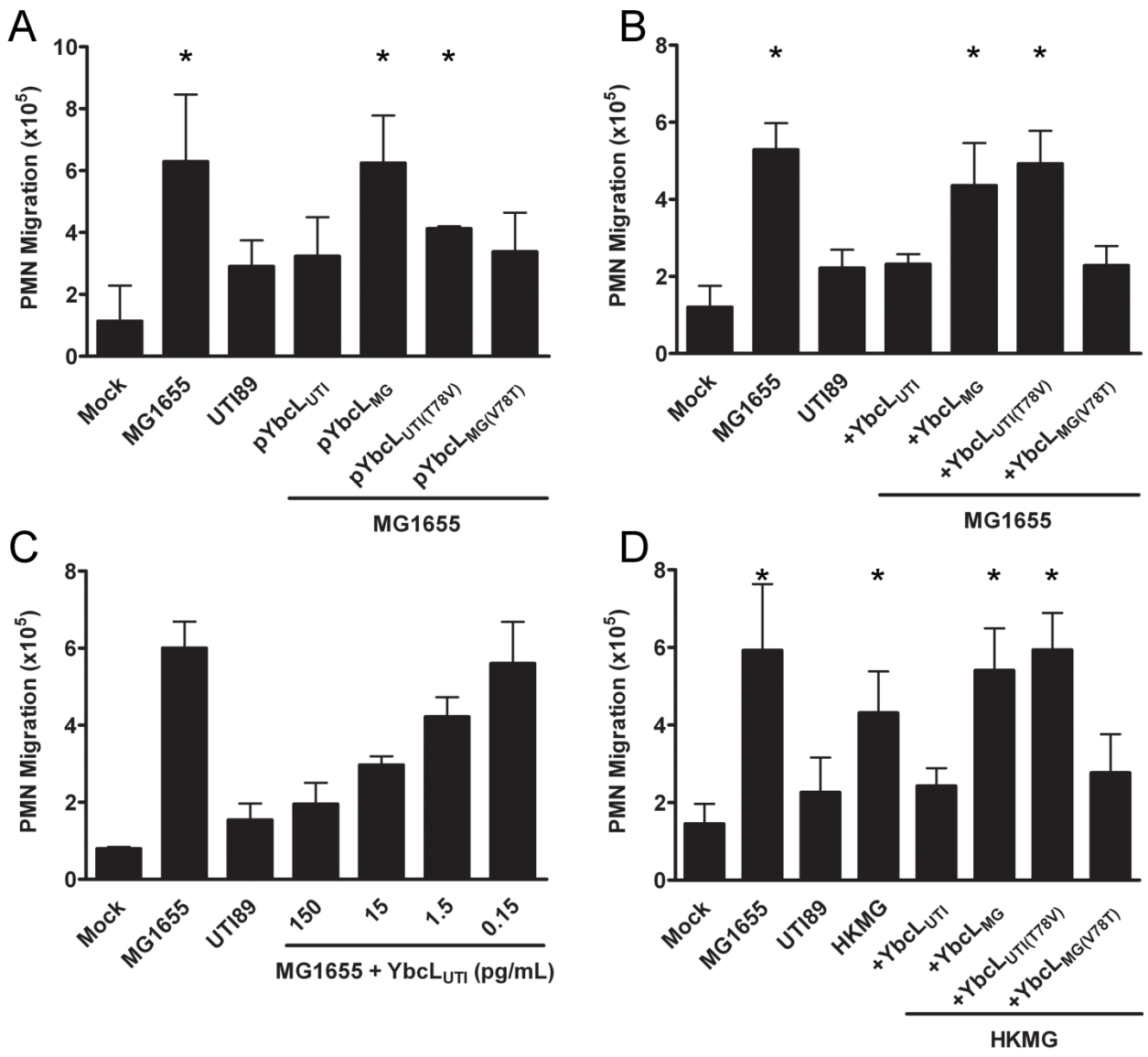

Figure 3: $YbcL_{UTI}$ confers suppressive activity on nonpathogenic *E. coli*. (A) The suppression of PMN migration by MG1655 episomally expressing the YbcL variants was evaluated using the transepithelial PMN migration model. This model was also used to assess changes in PMN migration caused by the addition of purified YbcL variants to the bacterial stimulus, live MG1655 (B, C) or heat-killed MG1655 (HKMG) (D). Purified YbcL variants were added to the bacterial stimulus immediately before infection of the epithelial layer at a final

concentration of 225 ng/ml unless otherwise indicated. YbcL variants containing a threonine at position 78, $YbcL_{UTI}$ and $YbcL_{MG(V78T)}$, suppressed PMN migration, while YbcL variants containing a valine at this position, $YbcL_{MG}$ and $YbcL_{UTI(T78V),}$ had no effect on PMN migration. Asterisks in panels A, B, and D indicate statistically significant ($p < 0.05$) increases in PMN migration compared to wild-type UTI89.

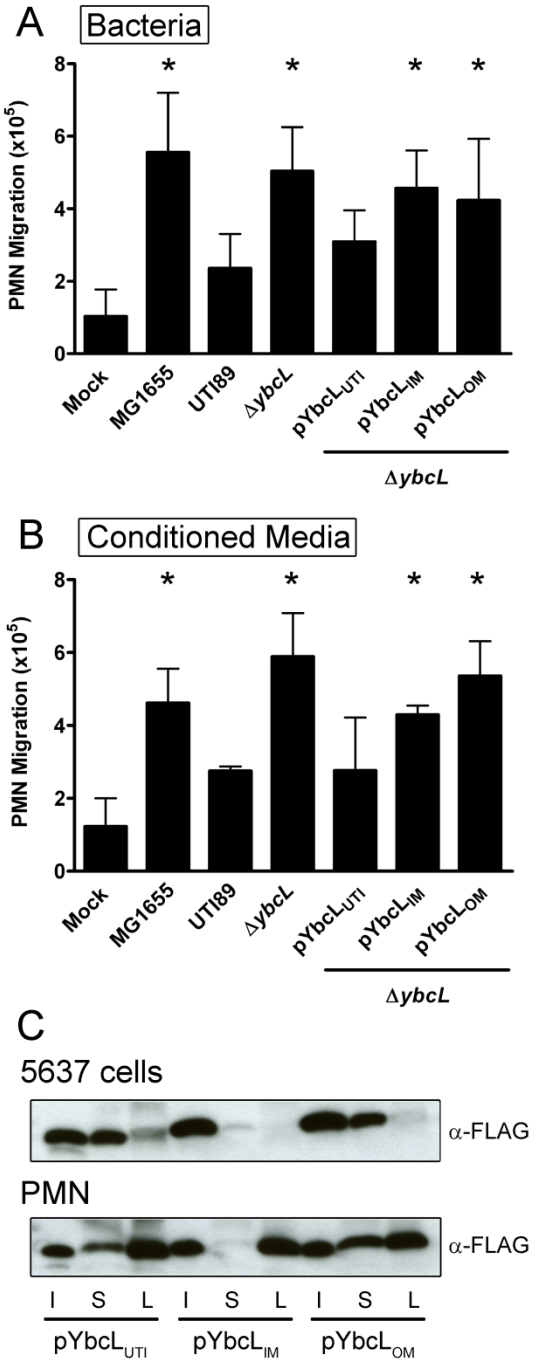

Figure 4: $YbcL_{UTI}$ is secreted. The level of PMN migration elicited by various strains of *E. coli* (A) or conditioned media (B) was evaluated using the transepithelial PMN migration model. YbcL variants tethered to either the inner or outer bacterial membrane ($YbcL_{IM}$ or $YbcL_{OM}$,

respectively) were unable to suppress PMN migration when expressed episomally in UTI89 Δ*ybcL*. The trends in PMN migration observed with conditioned media mimicked those observed when the inoculum included live bacteria. Asterisks in panels A and B indicate statistically significant ($p < 0.05$) increases in PMN migration compared to wild-type UTI89. (C) Localization of YbcL variants was assessed by Western blot. After 1 h infection of 5637 cells or PMN with the indicated strains of *E. coli*, the supernatant (S) and eukaryotic cell lysate (L) fractions were filter sterilized, TCA precipitated and resolved by SDS-PAGE. During infection of either cell type, $YbcL_{UTI}$ and $YbcL_{OM}$ were clearly detected in the supernatant fractions, while $YbcL_{IM}$ was minimally detected in those fractions. All three variants were detected in the PMN lysate; however, only $YbcL_{UTI}$ was detected in the 5637 cell lysate. An equivalent volume of each bacterial inoculum (I) is shown for comparison across strains.

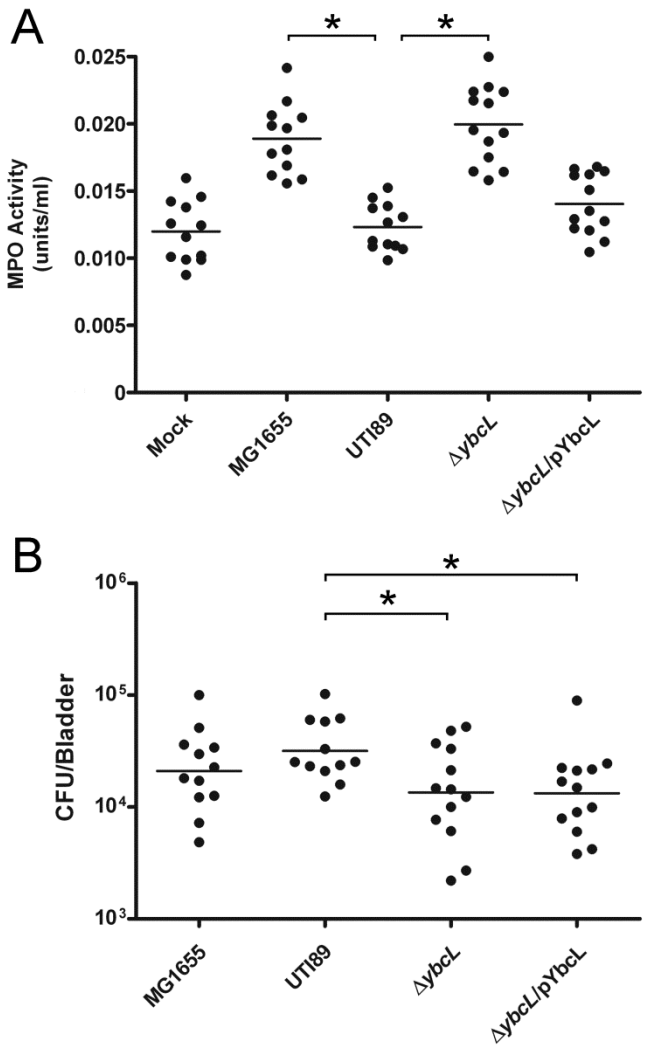

Figure 5: $YbcL_{UTI}$ suppresses acute PMN migration *in vivo*. C3H/HeN mice were infected with the indicated strains of *E. coli* or PBS and bladders were harvested at 1 h p.i. (A) A surrogate for PMN infiltration in the bladder, myeloperoxidase (MPO) activity was measured in bladder homogenates by fluorescent detection of an MPO product and is represented in units/ml. MG1655 and UTI89 Δ*ybcL* elicited significantly more PMN than wild-type UTI89 (* $p < 0.0001$). (B) Bladders infected with UTI89 Δ*ybcL* or the complemented strain showed a small but statistically significant decrease in bacterial load compared to UTI89 (* $p < 0.05$). Horizontal lines indicate the means in both panels.

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CHAPTER 3

LIBERATION OF YBCL FROM THE BACTERIAL PERIPLASM

Abstract

Uropathogenic *Escherichia coli (*UPEC) suppress neutrophil migration across bladder epithelia *in vitro* and *in vivo* through the action of a conserved protein, YbcL. Using varied approaches, we demonstrated that YbcL was released from UPEC during infection of bladder epithelial cells and neutrophils and that liberation of YbcL from the periplasm was required for suppression of neutrophil migration. Here we report our initial findings on the mode of YbcL release. During *in vitro* infection of bladder epithelial cells, YbcL was released into the supernatant in soluble form. Bacterial cytoplasmic proteins, GroEL and RNA Polymerase α subunit, were also detected in the supernatant, suggesting partial lysis of the bacterial inoculum during infection. The involvement of a type 2 secretion system (T2SS) or a type 4 pilus system (T4PS) in YbcL release was excluded, as the level of YbcL in the supernatant was unaffected by mutation of the outer membrane components of these systems. Development of a β–lactamase (TEM-1) reporter assay facilitated the characterization of YbcL release. YbcL::TEM-1, a translational fusion, was released by UPEC into the supernatant in a dose-, time-, and bladder epithelial cell-dependent fashion. Another periplasmic fusion, Skp::TEM-1, was detected in the supernatant; however, a cytoplasmic fusion, Gst::TEM-1, was noticeably absent. Taken together, these findings begin to illuminate the manner by which YbcL is released from the periplasm during UPEC infection of eukaryotic cells.

Introduction

Uropathogenic *Escherichia coli* (UPEC) manipulate the acute inflammatory response in the bladder, favoring the formation of the intracellular niche that propagates the infection over bacterial clearance by immune cells. An essential aspect of the innate immune response, migration of polymorphonuclear leukocytes (PMN; neutrophils) across the bladder epithelium into the lumen, is suppressed by a conserved UPEC protein YbcL (1). YbcL contains a canonical signal sequence and is targeted initially to the periplasm by the Sec system located in the inner membrane. Using a number of different approaches, we previously demonstrated that YbcL was released from the periplasm during UPEC infection (1). In contrast to the wild-type YbcL variant, YbcL variants that were unable to leave the periplasm did not complement the *ybcL* mutation in the transuroepithelial PMN migration assay. Additionally, purified YbcL protein suppressed PMN migration elicited by live and heat-killed bacterial stimuli as well as a peptide chemoattractant. YbcL was not detected in the supernatant during logarithmic phase growth of UPEC in nutrient rich media. However, YbcL was detected in the supernatant during UPEC infection of cultured bladder epithelial cells and PMN. Taken together, these data demonstrate that YbcL functions to suppress PMN migration from outside the bacterial periplasm. However, the mode of delivery of YbcL remains unclear.

Secretion, the transport of molecules (e.g., DNA and proteins) from the interior of the bacterial cell to the external environment, is a fundamental process of bacterial survival as well as pathogenesis. Gram-negative bacteria employ a variety of complex secretion systems. For example, type three secretion systems (T3SS), sometimes referred to as injectisomes or molecular syringes, are multiprotein complexes that span the inner and outer bacterial membranes delivering proteins directly from the bacterial cytoplasm into eukaryotic cells (2). Type four secretion systems (T4SS) are homologous to conjugation machinery and can transport

both DNA and protein (3). In contrast to T3SS, T4SS transport proteins from either the cytoplasm or the periplasm across the outer membrane (4). Unlike other bacterial pathogens, UPEC do not encode T3SS or T4SS. Like T3SS, type one secretion systems (T1SS) are also composed of a contiguous channel that translocates proteins from the cytoplasm to the extracellular space in a single step (5). Discovered recently, type six secretion systems (T6SS) deliver proteins to either prokaryotic or eukaryotic cells in a contact-dependent manner (6). Preliminary work suggests that T6SS substrates transit the apparatus in a one-step mechanism that avoids periplasmic intermediates. For this reason, neither T1SS nor T6SS mediate release of YbcL. Autotransporters, or type five secretion systems (T5SS), are typically multi-domain proteins that rely on the Sec machinery to reach the periplasm. The C-terminus of the autotransporter, after insertion into the bacterial outer membrane, transports the passenger domain across the membrane where it is cleaved and released (7, 8). The crystal structure of YbcL demonstrates that it is a globular protein and is unlikely to be a member of the T5SS family (9).

Type two secretion systems (T2SS) transport periplasmic proteins across the outer membrane through the secretin, a complex composed of a multimeric protein (10, 11). Type four pilus systems (T4PS) are structurally and functionally related to T2SS and have been shown, in some cases, to function as secretion systems (12, 13). Both T2SS and T4PS have been implicated in UPEC pathogenesis (14). Lastly, outer membrane vesicles (OMVs) are liberated by all Gram-negative bacteria and have been shown to deliver effector proteins of several pathogens, including UPEC, to eukaryotic cells (15-17). OMVs form when portions of the outer membrane pinch off and are composed of periplasmic contents surrounded by a lipid outer membrane bilayer (15). These secretion systems, T2SS/T4PS and OMVs, can be distinguished

by a characteristic of the secreted proteins, exposed versus protected from external elements, which may have implications for protein stability and targeting, for example. T2SS, T4PS, and OMVs contribute to colonization of the urinary tract by UPEC (14, 17).

In this study, we report our initial findings on the mode of delivery of YbcL from the bacterial periplasm during UPEC infection. The wild-type YbcL variant was released into the supernatant in a soluble form but was not dependent upon the T2SS or T4PS known to be encoded by the UPEC strain UTI89. By Western blot, bacterial cytoplasmic proteins were also detected in the supernatant during UPEC infection of bladder epithelial cells. Using a β-lactamase reporter assay, we demonstrated that release of YbcL::TEM-1, a translational fusion, into the supernatant was dose-, time-, and bladder epithelial cell-dependent. Finally, Skp::TEM-1, another periplasmic fusion, was present in the supernatant, but Gst::TEM-1, a cytoplasmic fusion, was not. These results suggest that release of YbcL from the periplasm is not mediated by a canonical secretion system, but rather through the release of intracellular contents into the supernatant, possibly in a selective fashion.

Materials and Methods

Bacterial strains and culture.

E. coli strains were grown statically in Luria-Bertani (LB) broth for 18 h at 37°C. Chloramphenicol and isopropyl β-D-1-thiogalactopyranoside (IPTG) were added at 20 μg/ml and 100 μM, respectively, where indicated. UTI89 is a UPEC isolate from a patient with cystitis (18). UTI89 *ybcL::cat*, UTI89/pYbcL$_{UTI}$ *ybcL::cat*, and UTI89/pYbcL$_{OM}$ *ybcL::cat* were created as previously described (1). UTI89 *yheF::cat* was created by linear transformation of UTI89/pKM208 (19) with a fragment amplified from template plasmid pKD3 (20) using the

primers MEL296 and MEL297 (primer sequences are given in **Table 1**). UTI89 *hofQ::cat* was created similarly using primers MEL300 and MEL301. The deletions were verified by direct sequencing.

Plasmid pTRC99A-Chl was generated from plasmid pTRC99A by replacing the ampicillin resistance cassette with a chloramphenicol resistance cassette. Briefly, pTRC99A was amplified using primers MEL245 and MEL246, and the chloramphenicol resistance cassette was amplified from template plasmid pKD3 (20) using primers MEL247 and MEL248. The PCR products were digested with PacI and SpeI and then ligated. Transformed clones of *E. coli* Top10 (Invitrogen) were selected on chloramphenicol plates and tested by colony PCR and restriction enzyme digestion. To assess release of YbcL from the bacterial periplasm using a β–lactamase substrate, we generated a fusion protein between YbcL and TEM-1, a β–lactamase variant, including a FLAG tag at the C-terminus. The *ybcL* gene was amplified from UTI89 genomic DNA using primers MEL253 and MEL275 and digested with SacI and NotI; TEM-1 sequence was amplified from plasmid pBR322 using primers MEL278 and MEL284 and digested with NotI and BamHI. The digested PCR products were ligated into pTRC99A-Chl (Chl^r) that had been digested with SacI and BamHI. We also generated control fusion proteins, Skp::TEM-1 and Gst::TEM-1, that localize to the periplasm and cytoplasm, respectively. Using a similar strategy, *skp* and *gst* were amplified from UTI89 genomic DNA using primer sets MEL285, 273 and MEL261, 277, respectively. The PCR products were cloned into pTRC99A-Chl as described above. Accuracy of the resulting constructs, pYbcL::TEM-1, pSkp::TEM-1 and pGst::TEM-1, was confirmed by direct sequencing. Expression of the fusion proteins upon IPTG induction was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of bacterial lysates.

Tissue culture.

The 5637 bladder epithelial cell line (derived from bladder carcinoma; ATCC HTB-9) was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (Sigma) at 37°C in a humidified atmosphere with 5% CO_2 unless otherwise noted.

Protease protection and ultracentrifugation assays.

The protease protection assay was developed based on previously published protocols (17, 21). To generate sterile conditioned media containing YbcL variants, 5637 cells in 15-cm dishes were infected with UTI89/pYbcL$_{UTI}$ *ybcL::cat* or UTI89/pYbcL$_{OM}$ *ybcL::cat* at a MOI of 40 at 37°C. After 1 h, the supernatant, or conditioned media, was sterilized using syringe-driven filter units (0.22µm pore size, Millipore), and separated into four aliquots. Proteinase K (Sigma), Triton X-100, and phenylmethylsulfonyl fluoride (PMSF) (Sigma), a protease inhibitor, were added to the sterile conditioned media at 200 µg/ml, 0.1%, and 5 mM, respectively, where indicated. After incubation at 37°C for 45 min, protein was precipitated from the reactions using 15% trichloroacetic acid (TCA) (Sigma).

In parallel experiments, sterile conditioned media, generated as described above, was ultracentrifuged at 245,000 × *g* for 1 h at 4°C. Protein was precipitated from control conditioned media and the ultracentrifuged supernatant using 15% TCA (Sigma), while the ultracentrifuged pellet was resuspended using Laemmli sample buffer.

Western blotting.

Proteins were separated by SDS-PAGE using 9-12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 2% nonfat milk + 2% bovine serum albumin (BSA) (Sigma), blots were probed with mouse anti-FLAG (1:1000, Sigma), rabbit anti-GroEL (1:400000, Sigma) or mouse anti-RNA Polymerase α subunit (1:4000, Neoclone) antibodies followed by goat anti-mouse or goat anti-rabbit IgG antibodies (1:2000, Sigma) and were developed using Tropix CDP-Star (Applied Biosystems).

β-lactamase reporter assays.

5637 cells in 6-well plates were infected with the indicated strains of *E. coli* at a MOI of 40 at 37°C. After 1 h, the supernatants were cleared by centrifugation at 16,000 x g for 5 min, and then moved to clean microfuge tubes. The cleared supernatant was aliquotted into a 96-well plate, 200 µl per well in triplicate, and CCF2-FA (Invitrogen), a β–lactamase substrate, was added at a final concentration of 500 nM. The reactions were incubated in the dark at 37°C with shaking for 1 h, before fluorescence (excitation at 409 nm and emission at 447 nm and 520 nm) was measured using a microtiter plate reader (Synergy 2; BioTek). Fluorescence is represented as a ratio of 447 nm to 520 nm, and the mean and standard deviation of replicates is shown. Statistically significant differences were evaluated using an unpaired Student's *t* test.

In analogous experiments, 5637 cells in 15-cm dishes were infected with the indicated strains of *E. coli* for 1 h at 37°C. The supernatant was sterilized using syringe-driven filter units (0.22µm pore size, Millipore), and concentrated 10-fold, from 9 ml to 0.9 ml, using centrifugal filter units according to the manufacturer's instructions (10 kDa, Millipore). CCF2 was added to 200 µl concentrated supernatant and fluorescence was measured after 1 h at 37°C as described above. The mean and standard deviation of replicates is shown.

Results

YbcL is released into the supernatant in soluble form.

To explore the mode of delivery of YbcL from the bacterial periplasm, we examined the characteristics of the extracellular fraction of YbcL. To clarify the relationship between YbcL localization and suppression of PMN migration, we included a bacterial outer membrane-tethered YbcL variant in these analyses (1). 5637 cells were infected with the *ybcL* mutant expressing either the wild-type UTI89 YbcL variant, $YbcL_{UTI}$, or the outer membrane-tethered YbcL variant, $YbcL_{OM}$, at a MOI of 40 for 1 h. The supernatant, or conditioned media, was filter sterilized and subjected to protease (Proteinase K) treatment in the presence and absence of detergent (Triton X-100) and protease inhibitor (PMSF). Protein was precipitated from the reactions using 15% TCA and resolved using SDS-PAGE. The wild-type YbcL variant was susceptible to digestion by protease in the absence of detergent (**Figure 1A**). In contrast, the outer membrane-tethered YbcL variant was present in 2 distinct fractions: one fraction was susceptible to degradation by protease in the absence of detergent, while the second fraction was only susceptible in the presence of detergent (**Figure 1A**). Levels of both YbcL variants were restored upon addition of protease inhibitor. The concentration of Proteinase K used in these reactions did not compromise OMV integrity in the absence of detergent, assessed using purified OMVs (data not shown).

To confirm these observations using an alternative approach, we ultracentrifuged sterile conditioned media, at a speed previously determined to pellet OMVs, to separate soluble and OMV fractions. The pellet, or insoluble fraction, was resuspended in Laemmli buffer, while protein remaining in the supernatant after ultracentrifugation, the soluble fraction, was precipitated using 15% TCA. Samples were resolved as described above. $YbcL_{UTI}$ was found

exclusively in the ultracentrifuged supernatant, while $YbcL_{OM}$ was found in both the ultracentrifuged supernatant and pellet (**Figure 1B**). The fraction of $YbcL_{OM}$ that is resistant to protease digestion in the absence of detergent and is present in the ultracentrifuged pellet is consistent with OMV cargo. In contrast, these data indicate that the wild-type YbcL variant is released into the supernatant in free, soluble form and is not packaged within OMVs.

Bacterial intracellular contents are released during infection of bladder epithelial cells.

The origin of $YbcL_{OM}$ in the conditioned media that was susceptible to protease in the absence of detergent and present in the ultracentrifuged supernatant was unclear, given the covalent bond tethering $YbcL_{OM}$ to the inner face of the bacterial outer membrane. We hypothesized that a low level of bacterial lysis occurred during infection of the bladder epithelial cells and that bacterial lysis supplied this fraction of $YbcL_{OM}$. To address this hypothesis, we infected bladder epithelial cells with UTI89/p$YbcL_{UTI}$ *ybcL::cat* as described above and resolved the filter sterilized, TCA precipitated supernatant by SDS-PAGE. In addition to $YbcL_{UTI,}$ bacterial cytoplasmic proteins GroEL and the α subunit of RNA Polymerase were detected in the supernatant (**Figure 2**). To ensure that the cytoplasmic proteins present in the supernatant were the result of bacterial lysis and not carryover from the bacterial overnight culture, the bacterial inoculum was washed repeatedly in PBS before infection of the bladder epithelial cells. The presence of bacterial cytoplasmic proteins in the supernatant may suggest that UPEC lyse and release internal contents, including $YbcL_{UTI}$, during infection of bladder epithelial cells.

To support this hypothesis and eliminate a role for the T2SS or T4PS in release of YbcL from the periplasm, we generated insertional mutations in the outer membrane secretin components of these systems in UTI89, *yheF* and *hofQ*, respectively. We found that the level of

YbcL in the supernatant, assessed by Western blot of filter sterilized, TCA precipitated supernatant, was not decreased in the absence of either secretin (data not shown). These results were confirmed using the β-lactamase reporter assay described below (data not shown). These data suggest that neither the T2SS nor the T4PS encoded by UTI89 mediates delivery of YbcL into the supernatant and support the hypothesis that YbcL may be released via bacterial lysis.

YbcL is released from the periplasm in a dose-, time- and bladder epithelial cell-dependent manner.

To further examine release of YbcL into the supernatant by UPEC, we developed an alternative, quantitative assay with increased accuracy and reproducibility that relies on the enzyme activity of β–lactamase (TEM-1). By generating a translational fusion between full-length YbcL and the mature form of TEM-1 (denoted YbcL::TEM-1), the level of β–lactamase activity in the supernatant can be used as a surrogate for the amount of YbcL::TEM-1 present. To detect β–lactamase activity, we chose a fluorescent substrate, CCF2, which consists of a cephalosporin core linking a 7-hydroxycoumarin to a fluorescein. In the absence of β-lactamase activity, excitation of the coumarin at 409 nm results in FRET to the fluorescein which emits a green signal (520 nm). In the presence of β-lactamase activity, CCF2 is cleaved, spatially separating the two fluorophores such that excitation of the coumarin results in emission of a blue fluorescent signal (447 nm). In accordance with previous experiments, 5637 cells in 6-well plates were infected with UTI89 pYbcL::TEM-1 at a MOI 40 for 1 h, unless otherwise indicated, before the supernatant was cleared by centrifugation and aliquotted into a 96-well plate. CCF2 was added to each well at a final concentration of 500 nM. Fluorescence was measured after 1 h at 37°C and is represented as a ratio of blue to green fluorescence (447 nm/520 nm).

5637 cells were infected with UTI89 pYbcL::TEM-1 induced overnight with varying concentrations of IPTG. Measurement of β-lactamase activity in the cleared supernatant revealed that the level of fluorescence positively correlated with the level of IPTG induction (**Figure 3A**). Therefore, the extent to which YbcL::TEM-1 is released into the supernatant is dependent upon the amount present in the bacterial periplasm. When 5637 cells were infected with UTI89 pYbcL::TEM-1 for varying times before the supernatant was cleared, the level of fluorescence in the supernatant increased with increasing duration of infection (**Figure 3B**). These data suggest that liberation of YbcL::TEM-1 from the bacterial periplasm occurs over time. Finally, UTI89 pYbcL::TEM-1 was incubated in the presence and absence of 5637 cells for 1 h before the level of YbcL::TEM-1 in the supernatant was evaluated using the β-lactamase substrate. Significantly higher levels of fluorescence were detected in supernatant generated in the presence of bladder epithelial cells compared to supernatant generated in the absence of epithelial cells (**Figure 3C;** $p < 0.0001$). These data suggest that release of YbcL::TEM-1 occurs either as a bacterial response to or as a consequence of exposure to bladder epithelial cells. Taken together, these data demonstrate that YbcL::TEM-1 is released from the periplasm in a dose-, time- and bladder epithelial cell-dependent manner.

Release of bacterial intracellular contents may be selective.

The presence of bacterial cytoplasmic proteins in the supernatant suggested that intracellular contents were released, possibly via lysis, during UPEC infection of 5637 cells. To evaluate the ability of the β-lactamase reporter assay to detect the release of intracellular contents, we generated 2 additional fusion proteins, Skp::TEM-1 and Gst::TEM-1, that localize to the bacterial periplasm and the cytoplasm, respectively, and expressed these fusions in UTI89.

Initial attempts to detect Skp::TEM-1 and Gst::TEM-1 in cleared supernatant after 1 h infection of 5637 cells in 6-well plates using CCF2 were unsuccessful (data not shown). The absence of a fluorescent signal could indicate that these fusion proteins were not released into the supernatant or that their levels in the supernatant were too low to detect using the initial experimental parameters. To distinguish between these possibilities, we modified the protocol.

5637 cells in 15-cm dishes were infected with the indicated strains of *E. coli* at a MOI of 40 for 1 h. Then, the supernatant was filter sterilized and concentrated 10-fold using centrifugal filter units with a 10 kDa cutoff. Control supernatant (input) and concentrated supernatant (retentate) were assayed for β-lactamase activity using CCF2 as described above. Upon concentration, Skp::TEM-1 was detected in the supernatant, although the level of fluorescence was lower than in retentate containing YbcL::TEM-1 (**Figure 4**). This disparity is likely due to differences in expression of the 2 fusion proteins (data not shown). In contrast, Gst::TEM-1 was not detected in the supernatant even after this 10-fold concentration (**Figure 4**). The absence of Gst::TEM-1 from the supernatant cannot be explained by low expression levels as Gst::TEM-1 expressed to levels similar to YbcL::TEM-1. In agreement with the β-lactamase reporter assay, Skp::TEM-1 and YbcL::TEM-1, but not Gst::TEM-1, were detected in filter sterilized, TCA precipitated supernatant by Western blot (data not shown). The presence of periplasmic proteins, Skp and YbcL, and the absence of cytoplasmic protein, Gst, from the supernatant suggests that the release of intracellular contents from bacteria may be selective. Future work will attempt to clarify these observations and elucidate the mechanism underlying release of YbcL from the bacterial periplasm.

Discussion

In this study, we investigated the mode of release of YbcL from the bacterial periplasm during UPEC infection of bladder epithelial cells. We demonstrated that YbcL was released into the supernatant in soluble form. Given this finding, we developed a β-lactamase reporter assay to further probe the dynamics of YbcL release. We demonstrated that the level of YbcL in the supernatant was influenced by the amount of YbcL in the periplasm, the duration of the infection, and the presence or absence of bladder epithelial cells. Additionally, we were able to detect a second, unrelated periplasmic protein in the supernatant. However, detection of cytoplasmic proteins in the supernatant was variable. In total, these findings inform our understanding of how bacterial exoproteins, specifically YbcL, can be released into the extracellular milieu. Additional work is required to clarify these observations.

O'Brien and colleagues demonstrated that cytotoxic necrotizing factor 1 (Cnf1) was initially targeted to the periplasm, subsequently packaged into OMVs, and then delivered to PMN (17). In contrast to purified Cnf1, Cnf1 in complex with OMVs attenuated PMN function, suggesting that delivery via OMVs was required for Cnf1 to reach the eukaryotic cytoplasm where it exerts its effect (17). In contrast, wild-type YbcL did not appear to be packaged into OMVs, as it was susceptible to protease in the absence of detergent and remained in the soluble fraction after ultracentrifugation. The outer membrane-tethered YbcL variant, on the other hand, appeared to be at least partially packaged within OMVs, as a fraction of $YbcL_{OM}$ was resistant to protease in the absence of detergent and was detected in the pellet after ultracentrifugation. As $YbcL_{OM}$ was unable to complement the *ybcL* mutation in the transuroepithelial PMN migration assay (1), association with OMVs, in this case, may preclude YbcL activity. As OMV cargo, $YbcL_{OM}$ may be inaccessible to binding partners or unable to reach its final destination possibly due to the lipid tether. $YbcL_{UTI}$ was detected in the supernatant and in bladder epithelial cell

lysate during UPEC infection, while $YbcL_{OM}$ was present only in the supernatant (1). The mode of delivery of $YbcL_{UTI}$ did not aid in the determination of site of action. Additional experiments will attempt to clarify whether YbcL localization to the supernatant or association with eukaryotic cells is required for suppression of PMN migration.

While one fraction of $YbcL_{OM}$ in the supernatant was consistent with OMV cargo, the origin of the second fraction was unclear. The presence of bacterial cytoplasmic proteins, GroEL and RNA Polymerase α subunit, in the supernatant suggested that intracellular contents were released during infection of bladder epithelial cells. We hypothesize that the release of intracellular contents, possibly through bacterial lysis, is responsible for the presence of $YbcL_{UTI}$ and $YbcL_{OM}$ in the supernatant. To support the hypothesis that bacterial lysis was solely responsible for $YbcL_{UTI}$ in the supernatant by excluding other secretion systems, we performed densitometry on the Western blots, comparing the level of GroEL in the supernatant to the level present in the input. Using these calculations and the number of CFU in the input, we estimate that less than 0.1% of the bacterial inoculum lysed during the 1 h infection. The ratio of YbcL in the supernatant to YbcL in the input was less than the same ratio calculated for GroEL, suggesting that YbcL was released into the supernatant solely via bacterial lysis and not through an alternative or additional mechanism. Given the low level of YbcL required to suppress PMN migration in the *in vitro* transuroepithelial PMN migration assay (<10 pM) (1), lysis of a minority of the bacterial inoculum may be sufficient to release an effective amount of YbcL.

To support the hypothesis that YbcL release is mediated by bacterial lysis, we examined the potential involvement of other secretion systems. We demonstrated previously that the level of the wild-type YbcL variant in the supernatant was similar independent of the bacterial strain used in the infection, MG1655 $pYbcL_{UTI}$ or UTI89/$pYbcL_{UTI}$ *ybcL::cat* (1), suggesting that the

mode of delivery of $YbcL_{UTI}$ is conserved between nonpathogenic and uropathogenic *E. coli*. By generating mutations in the secretins of the T2SS and T4PS, loci that are highly conserved between MG1655 and UTI89 (14), we demonstrated that neither secretion system mediated YbcL release. These data demonstrate that YbcL does not access the extracellular space via any of the canonical secretion systems.

To further our understanding of the parameters of YbcL release, we developed a reporter assay that relies on the detection of β-lactamase activity in the cleared supernatant using a fluorescent substrate. Compared to examining YbcL levels in filter sterilized, TCA precipitated supernatant by Western blot, the reporter assay is quantitative and highly reproducible. Using a translational fusion composed of YbcL and TEM-1, a β-lactamase variant, we demonstrated that release of YbcL::TEM-1 into the supernatant was dependent upon the concentration of the fusion present in the periplasm and increased over time. Furthermore, we demonstrated that release of YbcL::TEM-1 was significantly higher in the presence of bladder epithelial cells than in their absence. Using the β-lactamase reporter assay to examine the hypothesis that bacterial lysis mediates YbcL release, we found that Skp::TEM-1 was present in the supernatant, but Gst::TEM-1 was not. The release of intracellular proteins did not correlate with overall expression, nor with molecular weight as the translational fusions are equivalent in size (50 kDa). These results may suggest that release of intracellular contents is selective, although it is unclear how selectivity would be achieved during bacterial lysis. Additional experimentation is required to understand these seemingly incongruent pieces of data. The β-lactamase reporter assay will be invaluable in further exploring release of YbcL from the bacterial periplasm during infection.

The results presented herein suggest that YbcL is not released from the bacterial periplasm through a canonical secretion system or via OMVs. YbcL appears to be released along with other intracellular proteins in a dose-, time-, and bladder epithelial cell-dependent process. Although we cannot exclude the possibility that these observations are the result of multiple processes acting simultaneously, we hypothesize that the release of YbcL is mediated by bacterial lysis. Future work will attempt to determine the aspects of bladder epithelial cell exposure that stimulate or mediate YbcL release from the periplasm.

Acknowledgements

This work was supported by National Institutes of Health (NIH) grants R01-DK080752, R03-DK076556, and P50-DK064540. We thank K. Tiemann for technical assistance.

Tables and Figures

Table 1: Primers used in this study

Primer	Sequence (5' → 3')
MEL245	GCATACTAGTGGATCTAGGTGAAGATCCTTTTTGATAATCTC
MEL246	GCATTTAATTAACGCAAAAAGGCCATCCGTCAG
MEL247	GCATTTAATTAAGTGTAGGCTGGAGCTGCTTC
MEL248	GCATACTAGTCATATGAATATCCTCCTTAG
MEL253	GCATGAGCTCGGTCACAACAATGAGGTTTTTATG
MEL261	GCATGAGCTCATGAAATTGTTCTACAAACCGGGCGCCTGC
MEL273	GCATGCGGCCGCTTTAACCTGTTTCAGTACGTCGGCAGTG
MEL275	GCATGCGGCCGCCTTTATCTCATAAACTGGTGTTATCTCAGC GG
MEL277	GCATGCGGCCGCCTTTAAGCCTTCCGCTGACAGCGC
MEL278	GCATGCGGCCGCGCACCCAGAAACGCTGGTGA
MEL284	GCATGGATCCTTACTTGTCATCGTCGTCCTTGTAGTCCCAATGCTTAATC AGTGA
MEL285	GCATGAGCTCGTGAAAAAGTGGTTATTAGCTGCAG
MEL296	GCCTTGTGCAGGACACGCTGAGAACGAACAATACGGCGCTGTGTAGGC TGGAGCTGCTTC
MEL297	GTCGATTCGCAGCTGTTGCTCCTGGCGGTAGCGGGTGTATCATATGAAT ATCCTCCTTAG
MEL300	GGTGTTGCAGGTGCTGGCTGAACAGGAGAAGTTGAACCTGGTGTAGGCT GGAGCTGCTTC
MEL301	GGGCGTGACTTCCATCCCCAGGACGGCCTCTTTAAATTCCCATATGAAT ATCCTCCTTAG

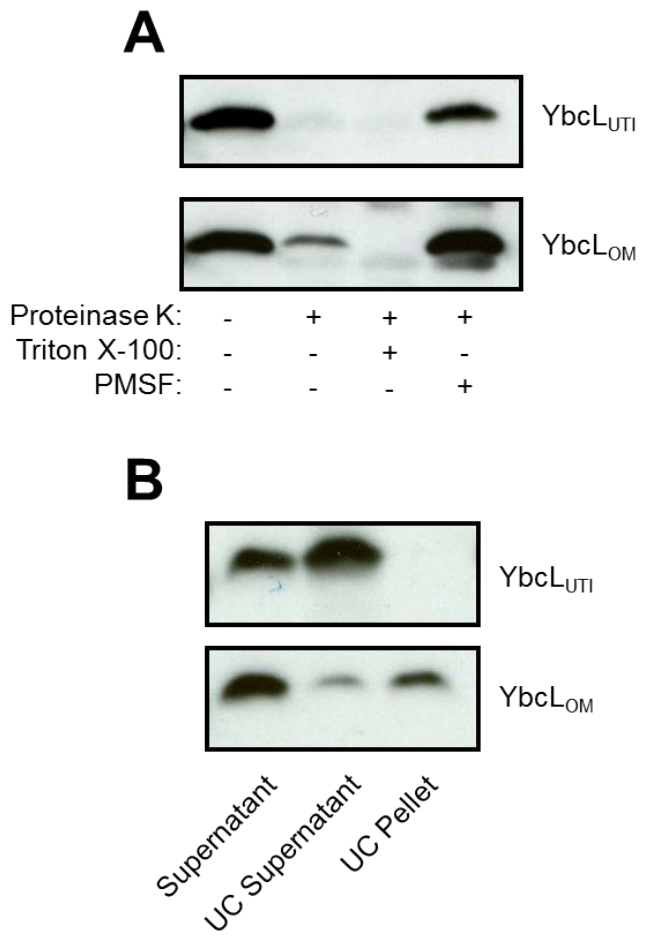

Figure 1: YbcL$_{UTI}$ is not packaged into OMVs. 5637 bladder epithelial cells were infected with UTI89/pYbcL$_{UTI}$ *ybcL::cat* or UTI89/pYbcL$_{OM}$ *ybcL::cat* for 1 h at 37°C, and then the supernatant, or conditioned media, was filter sterilized. (A) Proteinase K, Triton X-100 and PMSF were added to the supernatant, where indicated, and the reactions were incubated at 37°C for 45 min. (B) The sterile supernatant was ultracentrifuged at 245,000 × g for 1 h at 4°C. Proteins were TCA precipitated, when necessary, and resolved by SDS-PAGE. YbcL$_{UTI}$ was completely degraded by Proteinase K in the absence of Triton X-100, while YbcL$_{OM}$ was only partially degraded (A). YbcL$_{UTI}$ was detected exclusively in the supernatant, while YbcL$_{OM}$ was detected in both the supernatant and pellet after ultracentrifugation (B).

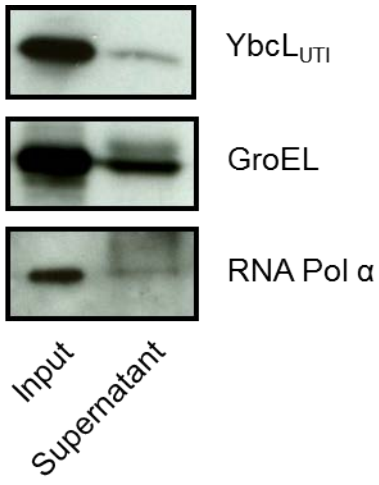

Figure 2: UPEC release intracellular contents during infection of bladder epithelial cells. 5637 cells were infected with UTI89/pYbcL$_{UTI}$ *ybcL::cat* as described above. The filter sterilized, TCA precipitated supernatant was resolved by SDS-PAGE and probed for YbcL$_{UTI}$ (FLAG), GroEL and RNA Polymerase α subunit. In addition to the periplasmic protein YbcL$_{UTI}$, bacterial cytoplasmic proteins, GroEL and the α subunit of RNA Polymerase, were detected in the supernatant.

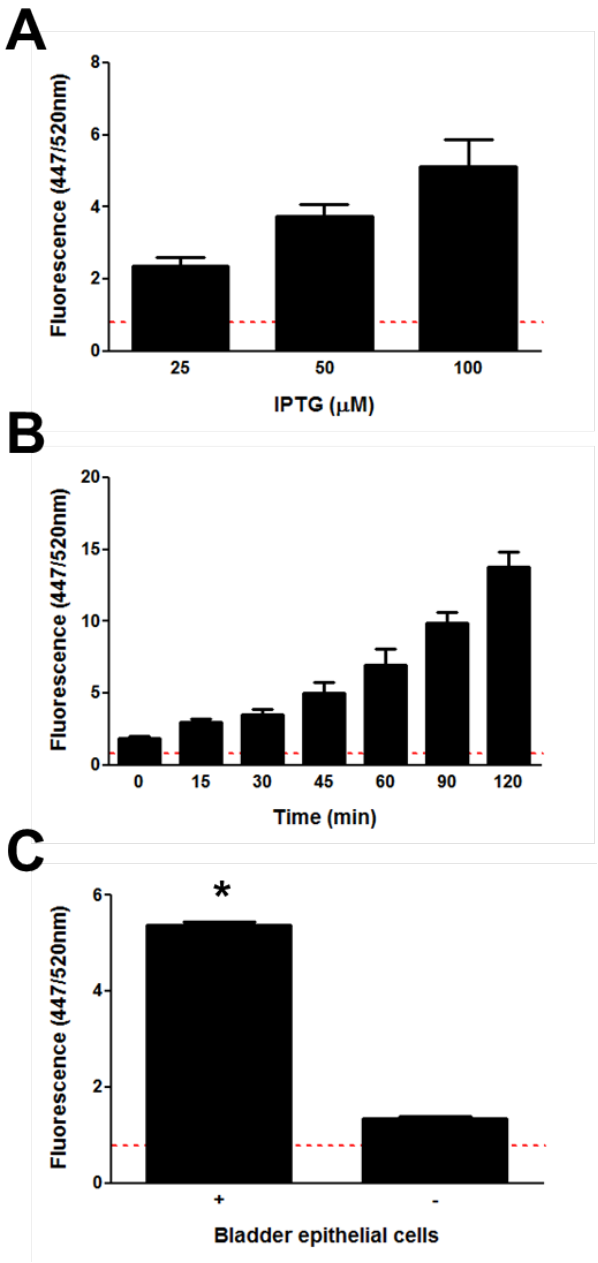

Figure 3: Liberation of YbcL is dose-, time- and bladder epithelial cell-dependent. 5637 cells were infected with UTI89 pYbcL::TEM-1 at a MOI 40 for 1 h unless otherwise indicated. The supernatant was cleared and aliquotted into a 96-well plate, and CCF2, a fluorescent β-lactamase substrate, was added to each well. After 1 h at 37°C, fluorescence was measured and is represented as a ratio of 447/520 nm. Background fluorescence in supernatant from infection

of 5637 cells with wild-type UTI89 is indicated by the red dashed line. The level of β–lactamase activity in the supernatant is dependent upon the concentration of YbcL::TEM-1 in the periplasm (A), the duration of the infection (B), and the presence of 5637 cells (* $p < 0.0001$) (C).

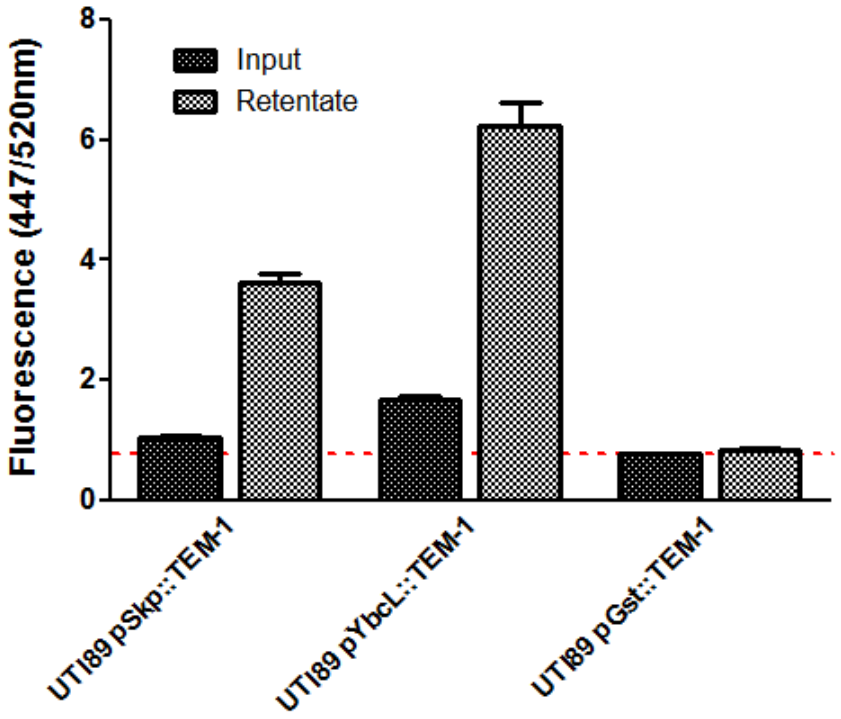

Figure 4: Selective release of bacterial intracellular proteins. 5637 cells in 15-cm dishes were infected with the indicated strains of *E. coli* at a MOI 40 for 1 h. The supernatant was filter sterilized and concentrated 10-fold using centrifugal filter devices. β-lactamase activity was measured in the control supernatant (input) and concentrated supernatant (retentate) using CCF2. Background fluorescence is indicated by the red dashed line. In contrast to Gst::TEM-1, Skp::TEM-1 and YbcL::TEM-1 were detected in the input and to a greater extent in the retentate.

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CHAPTER FOUR

EFFECTS OF YBCL ON BLADDER EPITHELIAL CELLS AND NEUTROPHILS

Abstract

Uropathogenic *Escherichia coli* (UPEC) manipulate the acute inflammatory response in the bladder, delaying the arrival of immune cells and facilitating invasion into the bladder epithelium, a requirement for the propagation of infection. Previously, we demonstrated that a protein encoded by UPEC, YbcL, suppressed the migration of polymorphonuclear leukocytes (PMN) or neutrophils across bladder epithelia *in vitro* and *in vivo*. To understand how UPEC employ YbcL to influence PMN migration, we examined a number of processes required for PMN to transit an epithelial barrier. We demonstrated that UPEC-mediated inhibition of the NF-κB pathway, which plays a pivotal role in initiating the pro-inflammatory response during infection, did not require YbcL. Production of PMN chemoattractants by bacteria and bladder epithelial cells, likely in an NF-κB-independent manner, was not disrupted in the presence of YbcL. Suppression of PMN migration by YbcL required a bladder epithelium on the Transwell inserts; YbcL did not inhibit PMN movement elicited by chemotactic molecules. Finally, integrity of the bladder epithelium was maintained even after high levels of PMN traversal, confirming that PMN migration in the *in vitro* model of transuroepithelial PMN migration is an active process. These findings contribute to our understanding of suppression of PMN migration by UPEC and further validate the *in vitro* model as a means to investigate events that occur *in vivo*.

Introduction

Mucosal surfaces of the human body are constantly exposed to external insults. The epithelial and resident immune cells that comprise the mucosa constitute the first line of defense again foreign threats such as bacterial pathogens. These cells are also responsible for initiating a pro-inflammatory response that elicits immune effector cells to aid in bacterial clearance. Infection of the bladder (i.e., cystitis) or kidneys (i.e., pyelonephritis), mucosal surfaces of the urinary tract, results in a characteristic inflammatory response. Aspects of the innate immune response to uropathogenic *Escherichia coli* (UPEC), the primary etiology of urinary tract infections (UTIs), have been elucidated using a variety of techniques and validated using samples from human patients. For example, interleukin (IL)-6, IL-8 and polymorphonuclear leukocytes (PMN; neutrophils) can be detected in the urine of patients with cystitis (1-3). Additionally, genetic polymorphisms in Toll-like receptors (TLRs) and IL-8 receptors within the human population influence disease outcome (4-6), further implicating these pathways in the innate immune response to UTI. Preliminary work has begun to characterize the acute inflammatory response in the bladder and to define how UPEC manipulate this response to cause disease. Given the complexity of these processes, further investigation is required.

In contrast to other mucosal surfaces like the gastrointestinal tract, the urinary tract, aside from the urethra, has been considered devoid of resident microbes and intolerant of bacterial colonization. Consequently, the presence of UPEC in the bladder lumen initiates a pro-inflammatory response. Specifically, bacterial products such as lipopolysaccharide (LPS), type I and P pili, and flagellin are recognized by pathogen recognition receptors (PRRs) including TLR4 and TLR5 expressed by bladder epithelial cells and resident immune cells (7-11). The engagement of PRRs results in the activation of pro-inflammatory signaling pathways such as the NF-κB and MAPK pathways. Sequential phosphorylation events result in the activation of

transcription factors that mediate the pro-inflammatory response through changes in gene expression. Among other things, cytokines and chemokines such as IL-6 and IL-8, respectively, are produced and secreted to form a chemoattractant gradient across the bladder epithelium (12, 13). In addition to chemotactic cytokines (e.g., IL-8), complement components (e.g., C5a), bioactive lipids (e.g., arachidonic acid metabolites) and bacterial products (e.g., N-formylated peptides) also participate in the formation of chemoattractant gradients (14). These chemically diverse molecules orchestrate the recruitment of immune cells, primarily neutrophils but also macrophages and monocytes, to the bladder.

The movement of PMN along a chemical gradient requires the temporal and spatial regulation of multiple intracellular signaling cascades that allow the cell to detect a gradient, polarize, and then migrate rapidly toward the highest concentration of the chemoattractant (15). The perception of chemoattractant substances by PMN is mediated primarily by G-protein coupled receptors (GPCRs) present in the cellular membrane. Upon ligand binding, GPCRs activate signaling pathways responsible for the reorganization of the cytoskeleton and membrane lipids. PMN exit the circulation via transendothelial migration and cross the extracellular matrix to reach the epithelium. To traverse the epithelial barrier, adhesins expressed by PMN (e.g., CD11b/CD18 and SIRPα) interact with adhesins expressed by epithelial cells (e.g., CD47, ICAM-1) in a step-wise process that allows PMN to first interact with the basolateral surface of the epithelium, navigate through cell-cell junctions, and then remain attached to the apical face of the epithelium or detach into the lumen (14, 16, 17). The serial engagement of adhesins is thought to initiate signaling cascades that facilitate this process. Once in the lumen, bacterial products stimulate the antimicrobial activities of PMN including phagocytosis, degranulation and ROS production (18). Much of the work defining PMN transit of epithelial barriers has been

conducted in the gastrointestinal tract and lung. Many of the mechanisms underlying these complex processes, especially in the urinary tract, are incompletely defined.

In vitro and *in vivo* models of cystitis have demonstrated that infection with nonpathogenic *E. coli*, laboratory or commensal strains, results in a robust inflammatory response at early time points (19). In contrast, UPEC elicit a muted inflammatory response as these strains have evolved mechanisms to manipulate processes within bladder epithelial and immune cells (19-22). Compared to nonpathogenic *E. coli*, UPEC delay the arrival of PMN to the bladder (19, 20), which affords a period without immune pressure during which UPEC can accomplish invasion into bladder epithelial cells. Suppression of PMN migration by UPEC has been demonstrated using an *in vitro* model of transuroepithelial PMN migration and an *in vivo* murine model of cystitis (19, 23). Additionally, we have demonstrated that a UPEC protein, YbcL, is involved in this phenotype, as a strain lacking *ybcL* expression is unable to suppress PMN migration (23). In spite of these findings, the mechanism underlying the low level of PMN migration elicited by UPEC and the role that YbcL plays in this phenotype remains unclear.

In this study, we examined multiple cellular processes involved in the recruitment of PMN across a bladder epithelium during UPEC infection. We demonstrated that UPEC inhibit NF-κB signaling independent of YbcL. Despite inhibition of the NF-κB pathway, chemoattractant molecules were produced during UPEC infection of bladder epithelial cells; the levels of chemoattractants were not influenced by YbcL. Importantly, YbcL was unable to inhibit PMN movement in the absence of a bladder epithelium. Finally, the integrity of the bladder epithelium was maintained even after high levels of PMN migration, demonstrating that traversal of the bladder epithelium in this model is not a passive process. Taken together, these

findings contribute to our understanding of PMN migration across a bladder epithelial barrier and the strategies employed by UPEC to influence this response.

Material and Methods

Bacterial strains and culture.

MG1655 is a K-12 laboratory strain of *E. coli* (24), and UTI89 is a UPEC isolate from a patient with cystitis (25). UTI89 *ybcL::cat* was created as previously described (23) by linear transformation of UTI89/pKM208 (26) with a product amplified from plasmid pKD3 (27). *E. coli* strains were grown statically in Luria-Bertani (LB) broth for 18 h at 37°C, and chloramphenicol was added at 20 µg/ml where indicated.

Tissue culture.

The 5637 bladder epithelial cell line (derived from bladder carcinoma; ATCC HTB-9) was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (Sigma) at 37°C in a humidified atmosphere with 5% CO_2 unless otherwise noted.

Western blotting.

5637 cells in serum-free RPMI media were infected with *E. coli* strains at a MOI 40 or mock infected for 2 h at 37°C. The cells were washed with PBS, and cell lysate was prepared by the addition of Laemmli sample buffer. Cell lysates were separated by SDS-PAGE using 9% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 2% nonfat milk + 2% bovine serum albumin (BSA) (Sigma), blots were

probed with mouse anti-β-actin, rabbit anti-NF-κB p65, or rabbit anti-phospho-NF-κB p65 (Ser536) (1:1000, Cell Signaling Technology), followed by goat anti-mouse or anti-rabbit IgG antibody (1:2000, Sigma), and were developed using Tropix CDP-Star (Applied Biosystems).

ELISA.

ELISAs were conducted as previously described (22). In short, 5637 cells were infected with *E. coli* at a MOI 40 or mock infected for 2 h at 37°C. Culture supernatants were removed, cleared by centrifugation at 16000 × *g* for 5 min, and stored at -80°C until IL-6 determination. ELISAs were conducted using Immulon 4 HBX microtiter plates (Thermo Scientific), anti-human IL-6 capture and detection antibodies and recombinant human IL-6 (R&D Systems), streptavidin-horseradish peroxidase (Zymed), 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma), and a microtiter plate reader (Synergy 2; BioTek). The mean and standard deviation from 3 independent experiments is shown.

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 according to a previously established protocol (19). 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 media to a concentration of 10^7 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).

Transuroepithelial PMN migration.

The transuroepithelial PMN migration assay has been previously described (19, 23). Briefly, ~10^5 5637 cells were seeded and grown on Transwell inserts (0.33cm^2 polycarbonate membranes with 3-µm diameter pores, Corning #3472) until the cells reached confluence, assessed by impermeability to liquid. Transwell inserts bearing confluent 5637 cells were inverted, and the epithelium was infected with *E. coli* at a MOI 40 or mock infected for 1 h. Transwell inserts were then righted into a 24-well plate (Ultra Low Attachment plates, Corning # 3473) and 10^6 freshly isolated human PMN, prepared as described above, were applied to the upper reservoir. After 1 h, the number of PMN in the lower reservoir was enumerated using a hemacytometer and is represented normalized to 10^6 input PMN. The mean and standard deviation from 3 independent experiments is shown.

Macromolecular permeability of the bladder epithelium.

To assess permeability of the bladder epithelium during the transuroepithelial PMN migration assay, 1 mg/ml fluorescein isothiocyanate-conjugated dextran (FITC-dextran) (10kDa, Invitrogen) was added to the PMN suspension before application to the upper reservoirs of the Transwell inserts. After 1 h, the level of FITC-dextran in the lower reservoir was quantified in triplicate using a microtiter plate reader (Synergy 2; BioTek) and is shown as a percentage of input FITC-dextran. The mean and standard deviation from 3 independent experiments is shown.

PMN migration.

To evaluate PMN movement in response to chemoattractant substances, 100 nM N-formyl-Met-Leu-Phe (fMLF; Sigma) was added to 0.6 ml serum-free RPMI in a 24-well low attachment plate. YbcL variants, $YbcL_{UTI}$ and $YbcL_{MG,}$ encoded by UTI89 and MG1655, respectively, were purified as previously described (23) and were added to the 24-well plate at a concentration of 225 ng/ml. Transwell inserts containing confluent 5637 epithelial layers or empty, uncoated Transwell inserts were added to each well and 10^6 PMN were applied to the upper reservoir. PMN migration across the Transwell insert into the lower reservoir was enumerated using a hemacytometer and is shown normalized to 10^6 input PMN. The mean and standard deviation from at least 3 independent experiments is shown.

Generation of conditioned media.

5637 cells grown to confluence in 10-cm dishes were infected with *E. coli* strains at a MOI 40 or mock infected for 1 h at 37°C in 5 ml serum-free RPMI. The supernatant was removed, sterilized using syringe-driven filter units (0.22μm pore size, Millipore) and 0.6 ml filter sterilized supernatant (conditioned media) was applied to the lower reservoir in the PMN migration assays described above. Conditioned media was also generated by incubating *E. coli* in serum-free RPMI in 10-cm dishes in the absence of 5637 cells.

Statistical analysis.

Statistically significant differences were evaluated using an unpaired Student's *t* test.

Results

UPEC inhibit NF-κB signaling independent of YbcL.

Despite the heterogeneous nature of UPEC, many UPEC strains suppress NF-κB signaling during *in vitro* infection of cultured bladder epithelial cells, evidenced by lower cytokine and chemokine levels (e.g., IL-6 and IL-8) in culture supernatants compared to cells infected with nonpathogenic *E. coli* (20-22). Given the prevalence of YbcL among UPEC isolates and the structural homology between YbcL and Raf kinase inhibitory protein (RKIP) (23, 28), a mammalian protein that inhibits numerous signaling cascades (29, 30), we hypothesized that YbcL functioned analogous to RKIP by suppressing NF-κB signaling during UPEC infection. To address this hypothesis, we infected 5637 bladder epithelial cells with various strains of *E. coli* for 2 h, and then probed the bladder cell lysates by Western blot for a subunit of NF-κB, p65, phosphorylated at serine 536, a post-translational modification that increases NF-κB activity (31). Infection with nonpathogenic *E. coli* MG1655 resulted in a modest increase in phospho-NF-κB p65, suggesting activation of the NF-κB pathway (**Figure 1A**). In contrast, infection with wild-type UTI89, a cystitis isolate, or UTI89 *ybcL::cat* resulted in lower levels of both the phosphorylated and unphosphorylated forms of NF-κB p65 (**Figure 1A**), suggesting abrogation of NF-κB signaling. To confirm these observations, we quantified IL-6 levels in culture supernatants, an indirect assessment of NF-κB activation. Infection with MG1655 resulted in significantly more IL-6 in the supernatant than infection with either wild-type UTI89 or the *ybcL* mutant (**Figure 1B;** $p < 0.05$). Furthermore, episomal expression of the UTI89 YbcL variant in MG1655 had no effect on the levels of phospho-NF-κB p65 or IL-6 (data not shown). Taken together, these data demonstrate that UTI89 inhibits NF-κB signaling at early time points independent of YbcL.

Bladder epithelial integrity is maintained during PMN migration.

Pathogenic *E. coli* that cause disease in the gastrointestinal tract (i.e., enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC)) secrete bacterial effectors that disrupt intercellular tight junctions (32), resulting in unregulated passage of immune cells into the gut lumen. We hypothesized that the differential PMN migration observed upon infection with MG1655 or UTI89 in the transuroepithelial PMN migration assay (19, 23) was the result of differences in bladder epithelial integrity. To examine the integrity of cell-cell contacts during traversal of the bladder epithelium by PMN, we assessed the permeability of the epithelial barrier to macromolecular flux using FITC-conjugated dextran (10 kDa). Bladder epithelial cells grown to confluence on Transwell inserts were mock infected or infected with various *E. coli* for 1 h before freshly isolated PMN and 1 mg/ml FITC-dextran were applied to the upper reservoir. Levels of PMN and FITC-dextran in the lower reservoir after 1 h were quantified using a hemacytometer and a microtiter plate reader, respectively. Consistent with prior results (23), infection with MG1655 resulted in significantly more PMN migration than mock infection or infection with wild-type UTI89 (**Figure 2A;** $p < 0.01$). Despite the differences in PMN migration, levels of FITC-dextran in the lower reservoir were similar independent of bacterial infection and bacterial strain (**Figure 2B**). Similar levels of FITC-dextran in the lower reservoir were observed upon infection with other UPEC strains that differ in their capacity to induce PMN migration (data not shown). These results demonstrate that the high level of PMN migration observed upon infection with MG1655 or UTI89 *ybcL::cat* (23) is not the result of compromised bladder epithelial integrity.

PMN migration is unaffected by YbcL in the absence of a bladder epithelium.

Of the strategies employed by bacterial pathogens to subvert the host immune response, manipulation of cellular processes required for cell movement is widespread (33). Given the high level of PMN migration observed upon infection with UTI89 *ybcL::cat* in the transuroepithelial PMN migration assay, we hypothesized that YbcL inhibited PMN movement. To address this hypothesis, we developed an *in vitro* assay that quantifies PMN migration across empty Transwell inserts containing pores sufficiently small to prevent passive diffusion in response to chemoattractant substances. Analogous to the transuroepithelial PMN migration assay, freshly isolated PMN were applied to the upper reservoir of an empty Transwell insert, and the level of PMN in the lower reservoir after 1 h was quantified using a hemacytometer. In the presence of a bladder epithelium on the Transwell inserts, fMLF, a chemotactic peptide, elicited significantly more PMN migration than mock infection (**Figure 3A;** $p < 0.05$). The addition of purified $YbcL_{UTI}$ to the lower reservoir, at a concentration 1500-fold above the lowest effective dose (23), significantly reduced the level of PMN migration (**Figure 3A;** $p < 0.05$). In agreement with previous findings, suppression of PMN migration by YbcL was observed upon addition of the UTI89 YbcL variant but not the MG1655 variant (23). When 5637 cells were excluded from the Transwell inserts, purified $YbcL_{UTI}$ was unable to suppress PMN migration elicited by fMLF (**Figure 3B**). These observations demonstrate that $YbcL_{UTI}$ does not inhibit PMN locomotion and that suppression of PMN migration by YbcL requires the presence of an epithelial layer.

YbcL does not influence the production of chemotactic molecules.

Immune cells, such as PMN, require chemical signals to direct their movement across epithelial barriers during infection. Consequently, bacterial pathogens have developed numerous

mechanisms for disrupting the production of chemoattractant gradients to prevent or delay the arrival of immune cells (34, 35). We hypothesized that the differential PMN migration observed in the transuroepithelial PMN migration assay upon infection with wild-type UTI89 or the *ybcL* mutant (23) was the result of differences in chemoattractant gradients generated by the bladder epithelia. To test this hypothesis, we assessed PMN migration across empty Transwell inserts in response to chemoattractant substances secreted by 5637 cells during infection. 5637 cells in 10-cm dishes were infected with *E. coli* or mock infected for 1 h, and then the supernatant, or conditioned media, was filter sterilized and added to the lower reservoir of empty Transwell inserts. Freshly isolated PMN were applied to the upper reservoir, and PMN migration into the lower reservoir was enumerated after 1 h using a hemacytometer. Sterile conditioned media generated during initial infection of 5637 cells with MG1655, UTI89 or UTI89 *ybcL::cat* elicited similar levels of PMN migration in the subsequent PMN migration assay (**Figure 4A**). We infer from this data that the quality and quantity of chemoattractant molecules present in the conditioned media are similar across conditions in order to elicit similar levels of PMN migration. This data demonstrates that YbcL does not influence the production of chemotactic molecules by cultured bladder epithelial cells.

In addition to chemoattractant production by the bladder epithelium, bacterial products may also direct PMN migration. To distinguish between 5637-derived and bacterial-derived chemotactic molecules in the conditioned media, *E. coli* were incubated in RPMI in the absence of 5637 cells in 10-cm dishes for 1 h, before the supernatant (conditioned media) was filter sterilized and applied to the lower reservoir of empty Transwell inserts. PMN migration from the upper reservoir into the lower reservoir was enumerated as described above. In accordance with previous results, conditioned media generated during incubation of MG1655, UTI89, or

UTI89 *ybcL::cat* in RPMI stimulated similar levels of PMN migration, independent of bacterial strain (**Figure 4B**). These data suggest that YbcL does not influence the production of bacterial-derived chemoattractant molecules. Taken together, these findings indicate that the differential PMN migration observed in the transuroepithelial PMN migration assay is not mediated by differences in chemotactic gradients composed of molecules produced by both bladder epithelial cells and bacteria.

Discussion

The present study examines numerous hypotheses on the mechanism underlying the low level of PMN migration observed upon infection with wild-type UTI89 in the transuroepithelial PMN migration assay. Given the increased PMN migration observed upon infection with UTI89 *ybcL::cat* (23), we focused our investigations on YbcL, although it is possible that suppression of PMN migration by UTI89 is a multi-faceted phenotype. We demonstrated that the low level of PMN in the lower reservoir upon UPEC infection was not due to the absence of a chemoattractant gradient; in fact, this phenotype was observed in spite of a chemoattractant gradient. As determined by examination of macromolecular permeability, the integrity of the bladder epithelium was not compromised by PMN migration. Finally, suppression of PMN migration by YbcL was not mediated by inhibition of PMN chemotaxis.

Inhibition of NF-κB signaling by UPEC isolates during infection of bladder epithelial cells has been widely reported (20-22, 36). Bacterial products including SurA and proteins involved in LPS biosynthesis (e.g., RfabE, WaaL, AmpG) were shown to contribute to this phenotype (22, 37). Recently, Dhakal and Mulvey also implicated the pore-forming toxin α-hemolysin (HlyA) in this phenotype. During UPEC infection of bladder epithelial cells,

activation of serine proteases, dependent upon HlyA, resulted in the degradation of proteins involved in numerous signaling cascades, including the NF-κB pathway (38). In agreement with these findings, infection with either wild-type UTI89 or UTI89 *ybcL::cat* resulted in lower levels of both the phosphorylated and unphosphorylated forms of NF-κB p65 compared to mock infection. Despite structural homology between YbcL and RKIP (28), a mammalian protein that inhibits the NF-κB pathway (30), YbcL had no effect on NF-κB signaling. Indeed, the lower levels of NF-κB p65 and phospho-NF-κB p65 upon UPEC infection were dependent upon HlyA, as infection with UTI89 Δ*hlyA* restored NF-κB p65 levels (M. Lau and D. Hunstad, unpublished data). From these data, we conclude that the differential PMN migration observed upon infection with wild-type UTI89 or the *ybcL* mutant in the transuroepithelial PMN migration assay is not the result of differences in the activation status of the NF-κB pathway.

As production of chemotactic molecules is only partly dependent upon NF-κB activation, we took a more general approach to investigate potential differences in chemoattractants produced during infection. We demonstrated that sterile conditioned media generated in the presence or absence of bladder epithelial cells elicited similar levels of PMN migration across empty Transwell inserts independent of *E. coli* strain. Given that nonpathogenic *E. coli* and UPEC express functionally distinct YbcL variants (23), we conclude that YbcL does not influence the production of chemotactic molecules by either bladder epithelial cells or bacteria. In support of these data and the observations on NF-κB activation, quantification of IL-8, a PMN chemoattractant, in the upper and lower reservoirs during the transuroepithelial PMN migration assay revealed no significant differences upon infection with UTI89 versus the *ybcL* mutant (M. Lau and D. Hunstad, unpublished data). These results suggest that the increased PMN migration observed upon infection with UTI89 *ybcL::cat* compared to wild-type UTI89 in this assay is not

due to augmented production of chemotactic molecules. Although all of the chemoattractants in these assays have not been identified, these results suggest that a chemoattractant gradient is produced, possibly in an NF-κB-independent manner, across the bladder epithelium during infection with UTI89 in the transuroepithelial PMN migration assay. It remains unclear how UPEC utilizes YbcL to prevent PMN from transiting the epithelium in response to these chemotactic signals.

In modifying the transuroepithelial PMN migration assay to remove the bladder epithelial barrier, we were able to examine the direct effect of purified YbcL on PMN movement in response to chemotactic molecules. In contrast to the suppressive effect observed in the presence of a bladder epithelium, YbcL had no effect on PMN migration across empty Transwell inserts in response to a peptide chemoattractant, fMLF. In agreement with these data, sterile conditioned media generated during initial infection of bladder epithelial cells with wild-type UTI89 suppressed PMN migration when a bladder epithelium was present on the Transwell insert (23), but not in the absence of a bladder epithelium. As YbcL was unable to influence PMN migration in the absence of an epithelial barrier even at very high concentrations, we conclude that YbcL does not inhibit PMN chemotaxis (e.g., polarization or cytoskeletal rearrangement) or the perception of chemotactic signals by PMN. Therefore, the low level of PMN migration observed upon infection with wild-type UTI89 in the transuroepithelial PMN migration assay is not the result of inhibition of PMN movement or prevention of chemotactic signal detection by YbcL. These data suggest that YbcL instead exerts its effect on the bladder epithelium, or on interactions between PMN and epithelial cells. Experiments aimed at addressing the site of action of YbcL have been largely unfruitful, likely because of the extremely low concentration of

YbcL required to suppress PMN migration. The development of assays with increased sensitivity will facilitate further investigation.

Pathogenic *E. coli* that cause disease in the gastrointestinal tract produce bacterial effectors that increase epithelial permeability through disruption of cell-cell contacts (32), facilitating movement of immune cells into the lumen. In contrast, probiotic bacteria enhance barrier function of the gastrointestinal epithelium, preventing pathogenic strains from invading underlying tissue and inhibiting immune cell migration (39). Given these findings in the gastrointestinal tract, we tested the hypothesis that the differential PMN migration observed in the transuroepithelial PMN migration assay was the result of differences in barrier function of the epithelium. We found that macromolecular permeability of the bladder epithelium did not correlate with the level of PMN migration, demonstrating that increased PMN migration observed upon infection with MG1655 or UTI89 *ybcL::cat* (23) was not due to increased epithelial permeability. These results serve as a contrast to pathogenic *E. coli* in the gastrointestinal tract. It is likely that precise rearrangement of cell-cell contacts during PMN traversal of the cultured uroepithelium prevents macromolecular flux into the lower reservoir, analogous to PMN migration across epithelial barriers *in vivo*. These observations further validate this *in vitro* assay as a means to investigate PMN movement through intercellular contacts of the bladder epithelium.

In this study, we investigated a role for YbcL in manipulating the processes required for PMN to transit the bladder epithelium. We interrogated activation of the NF-κB pathway, production of chemotactic molecules by bladder epithelial cells and bacteria, integrity of the bladder epithelium and, finally, PMN chemotaxis. We conclude that YbcL does not influence these processes. The transit of PMN through epithelia is a multi-step process that requires the

coordinated activity of many proteins and signaling cascades, many of which are incompletely defined. Additional work, utilizing the *in vitro* transuroepithelial PMN migration assay, focused on identifying these factors may illuminate additional steps in the cascade that are subject to inhibition by bacterial proteins such as YbcL.

Acknowledgements

This work was supported by National Institutes of Health (NIH) grants R01-DK080752 and P50-DK064540. We thank J. Loughman and K. Tiemann for technical assistance.

Tables and Figures

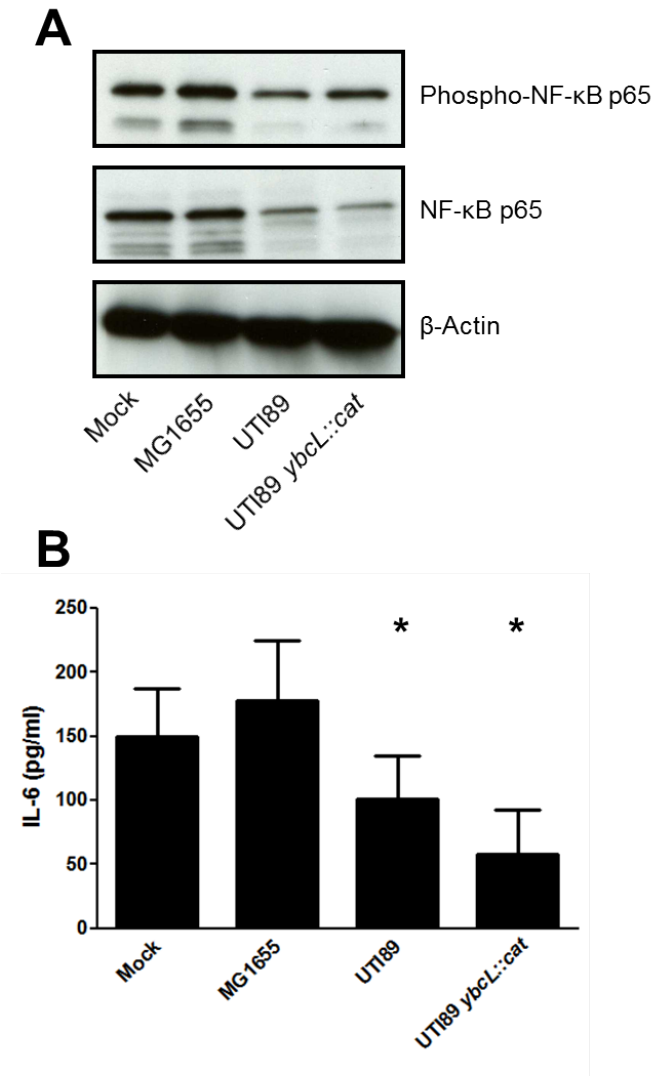

Figure 1: UTI89 inhibits NF-κB signaling independent of YbcL. 5637 bladder epithelial cells were infected with the indicated strains of *E. coli* or mock infected for 2 h. Cell lysates were probed for phospho-NF-κB p65 (Ser536), NF-κB p65, and β-actin by Western blot (A). Levels of IL-6 in culture supernatants were determined by ELISA (B). In contrast to MG1655, infection with wild-type UTI89 or UTI89 *ybcL::cat* resulted in lower levels of NF-κB p65, both phosphorylated and unphosphorylated forms, in bladder cell lysates and significantly lower levels of IL-6 in culture supernatants (* $p < 0.05$).

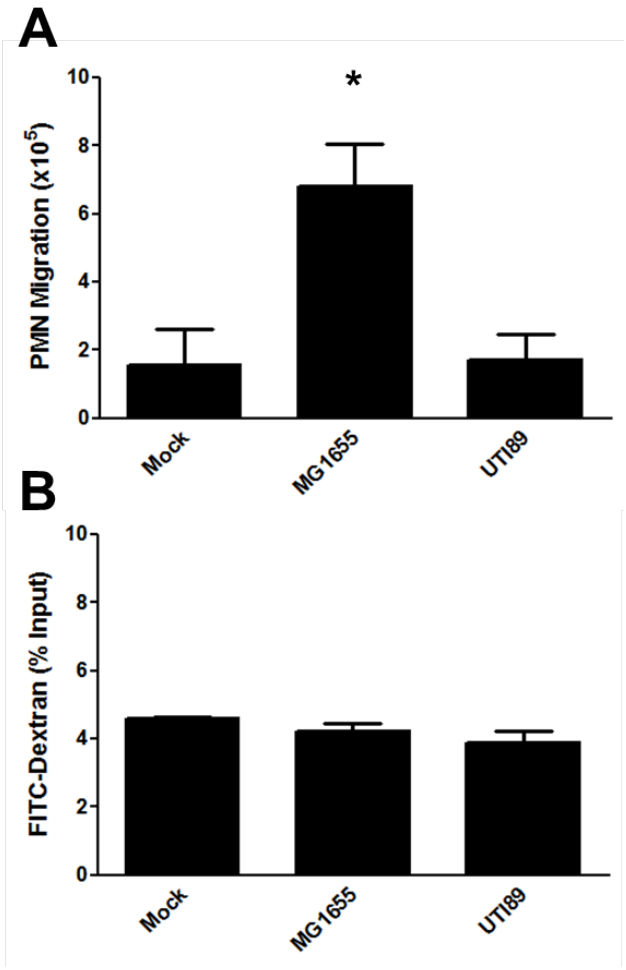

Figure 2: Epithelial integrity is maintained during PMN migration. 5637 bladder epithelial cells grown to confluence on Transwell inserts were infected with the indicated strains of *E. coli* or mock infected for 1 h. Freshly isolated PMN and FITC-labeled dextran were applied to the upper reservoir. After 1 h, the level of PMN migration into the lower reservoir was enumerated using a hemacytometer and is shown normalized to 10^6 input PMN (A). The level of FITC-dextran in the lower reservoir was quantified using a microplate reader and is shown as a percentage of input FITC-dextran (B). Infection with MG1655 elicited significantly more PMN migration than mock infection or infection with UTI89 (* $p < 0.01$). The level of FITC-dextran in the lower reservoir was similar across conditions, independent of the level of PMN migration.

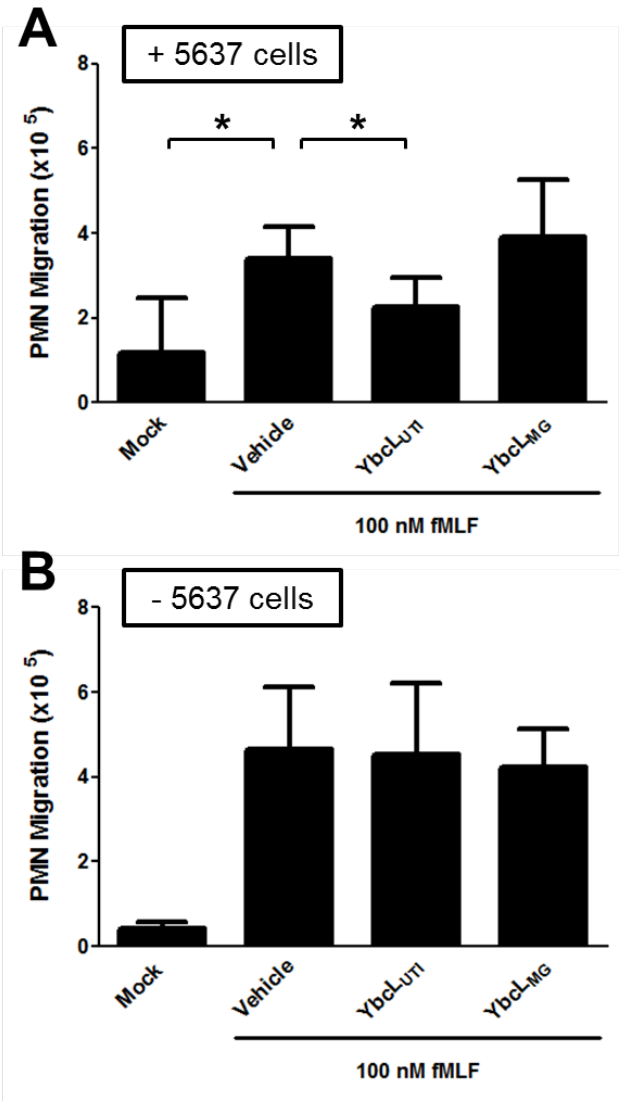

Figure 3: PMN movement is unaffected by YbcL. Freshly isolated PMN were applied to the upper reservoir of either Transwell inserts bearing confluent 5637 cell layers (A) or empty Transwell inserts (B) and migration into the lower reservoir was enumerated after 1 h, as described for Fig. 2. fMLF and purified YbcL variants were included in the lower reservoirs at concentrations of 100 nM and 225 ng/ml, respectively, where indicated. In the presence of a bladder epithelium on the Transwell inserts, $YbcL_{UTI}$, but not $YbcL_{MG}$, significantly reduced the level of PMN migration elicited by fMLF (* $p < 0.05$). In contrast, $YbcL_{UTI}$ had no effect on the level of PMN migration in the absence of a bladder epithelium on the Transwell inserts.

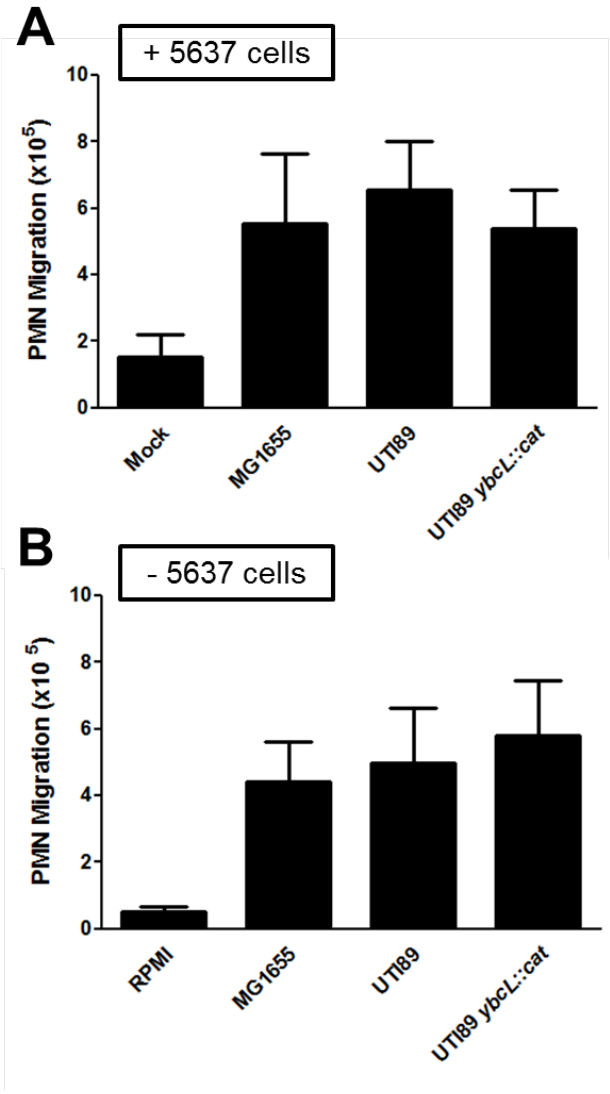

Figure 4: YbcL does not affect the production of chemotactic molecules by bladder epithelial cells or *E. coli*. Conditioned media was generated by incubating the indicated strains of *E. coli* in RPMI in the presence of 5637 cells (A) or in the absence of 5637 cells (B) for 1 h. The conditioned media was filter sterilized and applied to the lower reservoir of empty Transwell inserts. PMN were applied to the upper reservoir and migration into the lower reservoir was enumerated after 1 h, as described for Fig. 2. The levels of PMN migration elicited by conditioned media, generated either in the presence of 5637 cells or in the absence, were similar across bacterial strains.

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CHAPTER 5

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The overarching goal of this work was to further our understanding of uropathogenic *Escherichia coli* (UPEC)-mediated modulation of the innate immune response in the bladder. A transcriptional study identified *ybcL* as being up-regulated during UPEC infection of cultured bladder epithelial cells and human polymorphonuclear leukocytes (PMN; neutrophils), suggesting that the gene product was involved in adaptation of UPEC to these cell types. We subsequently demonstrated that YbcL suppressed PMN migration across a cultured bladder epithelium in an *in vitro* model of transuroepithelial PMN migration and an *in vivo* model of murine cystitis (i.e., bladder infection) (1). The bulk of this thesis work focused on investigating how a low abundance protein encoded by UPEC, YbcL, could have such a profound effect on the innate immune response in the bladder.

Allelic Variation in *ybcL* Loci

In examining YbcL homologs encoded by pathogenic and nonpathogenic *E. coli*, amino acid differences were identified. The presence of a threonine at position 78 in YbcL correlated with suppression of PMN migration in the transuroepithelial PMN migration assay (1). The majority of sequenced and clinical UPEC strains encoded a threonine-containing YbcL variant (1), demonstrating that suppression of PMN migration by YbcL is a conserved mechanism for modulating the innate immune response. In contrast, few nonpathogenic *E. coli* strains encoded a threonine-containing YbcL variant (1). It is not clear if valine- and alanine-containing YbcL variants confer an advantage to *E. coli* that warrants maintenance of these loci. Threonine at

position 78 in YbcL was highly conserved among clinical UPEC isolates associated with pyelonephritis (i.e., kidney infection) and asymptomatic bacteriuria (ASB) (i.e., bacteria in the urine in the absence of symptoms). In addition to influencing the innate immune response in the bladder, YbcL may also suppresses PMN recruitment to the kidneys, though suppression of PMN migration across kidney epithelial cells *in vitro* has not been assessed. The lack of a well-developed animal model that is specific for pyelonephritis would limit extension of such *in vitro* findings to an *in vivo* scenario. The conservation of T78 in YbcL among clinical isolates associated with ASB is puzzling, as the absence of an inflammatory response in ASB has been primarily attributed to colonization with less virulent bacteria rather than active suppression of inflammatory pathways. Studies investigating the dynamics of ASB may uncover a role for YbcL in this disease state.

YbcL is structurally homologous to Raf kinase inhibitory protein (RKIP) (2), a mammalian protein that inhibits numerous signaling cascades including the MAPK and NF-κB pathways (3, 4). Despite this structural homology, we demonstrated that YbcL does not inhibit signaling through these pathways. Phosphorylation of RKIP at serine 153 influences its affinity for binding partners (5, 6). Given the importance of T78 in YbcL and the structural homology to RKIP, YbcL may be phosphorylated at this residue, likely by a eukaryotic kinase. Mutation of the threonine at position 78 to a glutamic acid to mimic a phosphorylated threonine yielded a YbcL variant ($YbcL_{T78E}$) that was unable to suppress PMN migration (M. Lau and D. Hunstad, unpublished data). As this result is difficult to interpret, additional tools such as anti-phospho-threonine antibodies would be useful in further testing this hypothesis.

Recent findings have demonstrated that post-translational modification (i.e., phosphorylation at serine 153) of RKIP leads to homodimer formation, which in turn, affects

interactions with binding partners (7). The K12 YbcL variant crystallized as a homodimer (2). In monomeric form, the presumed ligand binding pocket of YbcL appears to be incomplete. In contrast, completion of the RKIP ligand binding pocket, which mediates interactions with binding partners, does not require dimerization, as monomeric RKIP contains an intact pocket (8). It is not clear if an intact binding pocket and/or dimerization are required for suppression of PMN migration by YbcL. In addition to investigating post-translational modification of T78 in YbcL, experiments addressing dimer formation by YbcL variants will be informative. As with RKIP, an amino acid distal to the binding pocket (T78) may affect the activity of YbcL by influencing its ability to oligomerize.

Threonine at position 78 was not required for release of YbcL from the bacterial periplasm, as a valine-containing variant was detected in the supernatant at levels similar to a threonine-containing variant. One hypothesis is that T78 is instead required for YbcL activity following release from the periplasm; for example, T78 may influence protein-protein interactions. RKIP has been shown to interact with phosphorylated protein targets, and we hypothesize that YbcL functions similarly. As YbcL was detected in the supernatant as well as in cell lysates prepared from UPEC-infected bladder epithelial cells and PMN, the exact site of action of YbcL is unclear. This finding in addition to the low concentration of YbcL required to suppress PMN migration complicates protein-protein interaction studies, discussed below. Additional experiments are required to address questions such as site of action and subcellular localization before protein interaction studies will yield fruitful results.

Release of YbcL from the Periplasm

Using a number of different approaches, we demonstrated that suppression of PMN migration by UTI89 required release of YbcL from the bacterial periplasm (1). Levels of YbcL and GroEL, a bacterial cytoplasmic protein, in the supernatant were similar independent of bacterial strain, MG1655 or UTI89 *ybcL::cat* (M. Lau and D. Hunstad, unpublished data), suggesting the mode of release is conserved between nonpathogenic and uropathogenic *E. coli*. In support of these data and the finding that liberation of YbcL is required for suppression of PMN migration, MG1655 episomally expressing the UTI89 YbcL variant elicited significantly fewer PMN than wild-type MG1655. This commonality allowed us to eliminate the scenario in which potential secretion machinery was not encoded by both strains.

Secretion of effectors or toxins by bacterial pathogens is a common strategy to influence eukaryotic processes. Many bacterial pathogens encode type 3 secretion systems (T3SS) and type 4 secretion systems (T4SS) to achieve these means. However, these complex machines are not encoded by UPEC. Recently, type 2 secretion systems (T2SS), type 4 pilus systems (T4PS) and outer membrane vesicles (OMVs) have been implicated in UPEC pathogenesis (9, 10). Because YbcL was released into the supernatant in soluble form and did not appear to be packaged in OMVs, we examined a role for the T2SS and T4PS found in both MG1655 and UTI89. The level of YbcL detected in the supernatant was unchanged upon deletion of either secretin, YheF or HofQ, demonstrating that neither secretion system is solely responsible for liberation of YbcL. The generation of a strain containing mutations in both secretins would be required to eliminate the possibility of functional redundancy between these systems. A second putative T2SS gene cluster encoded by UTI89 shares homology with a cluster encoded by enterotoxigenic *E. coli* (ETEC) strain H10407 (10). This gene cluster is absent from the clinical UPEC strain CFT073, isolated from the blood of a patient with pyelonephritis, and has extremely

low homology to the T2SS encoded by MG1655. Although it is unlikely that this second T2SS mediates release of YbcL, mutation of the secretin component, GspD, in UTI89 would address this uncertainty. Taken together, these findings suggest that liberation of YbcL from the bacterial cell is not mediated by the T2SS or T4PS.

Many integral membrane proteins found in the bacterial outer membrane are porins or transporters that regulate the movement of ions and solutes in and out of the periplasm. The pores or channels of these proteins are not large enough to allow passage of a fully folded protein, even one as small as YbcL. However, there are proteins and protein complexes that function to transport folded proteins across the bacterial outer membrane. For example, ushers of the chaperone/usher secretion pathway serve as platforms for pilus biogenesis, assembling and transporting pilus subunits through their large central channels (11). A number of chaperone/usher systems are encoded by UPEC, although few are found in MG1655. When pilus biogenesis is not active, the ushers are gated to prevent the unregulated flux of ions and solutes (12, 13). In addition, the assembly of pilin subunits is a highly ordered process that requires interactions between a cognate periplasmic chaperone and the N and C termini of the usher (14). Chaperone/usher systems have not yet been implicated in the secretion of proteins other than pili subunits. Given these observations, it is unlikely that ushers facilitate transport of YbcL across the bacterial outer membrane, though this hypothesis could be tested through the generation of usher mutants.

Similar to pili, the biogenesis of flagella requires a protein complex to move bacterial proteins across the outer membrane (15). Evolutionarily related to T3SS, flagella have been shown to secrete bacterial proteins into the supernatant, in addition to mediating motility (16). Composed of FlgH, the L-ring situated in the outer membrane forms a pore large enough to

transport folded proteins such as flagellin (FliC). In UTI89, the *ybcL* locus is surrounded by genes involved in flagella biosynthesis (17). In light of these observations, we examined the hypothesis that YbcL was released from the periplasm via flagella biosynthesis machinery. As the level of YbcL in the supernatant was unaffected by mutation of *flgH* (M. Lau and D. Hunstad, unpublished data), YbcL is not transported across the bacterial outer membrane by the flagellar L-ring. It is possible that additional outer membrane proteins, encoded by both MG1655 and UTI89, exist that could transport YbcL across the outer membrane into the supernatant.

Given the presence of cytoplasmic proteins GroEL and RNA Polymerase α subunit in the supernatant during UPEC infection, we hypothesized that release of YbcL from the periplasm was achieved through bacterial cell lysis. The genes encoding Shiga toxin (Stx1), *stxA* and *stxB*, are often found within prophages of the λ bacteriophage family (18). Stx1 is produced upon phage induction and is transported to the bacterial periplasm. The accumulation of Stx1 in the periplasm is controlled by phage-mediated bacterial lysis, which is also the mechanism of Stx1 release (19). In MG1655, the *ybcL* locus is found within a lambdoid-like prophage, DLP12 (20). This gene organization is not conserved in UTI89, although DLP12 prophage is present in the UTI89 genome. Nonetheless, we tested the hypothesis that YbcL was delivered in a manner analogous to Stx1 by examining a role for UTI89-encoded DLP12 prophage lysis proteins, YbcR2, YbcS2 and C5133, homologs of the lambda proteins S, R, and Rz/Rz (1), respectively, as mediators of bacterial lysis and release of YbcL from the periplasm. The level of YbcL detected in the supernatant was unaltered by deletion of these lysis genes (M. Lau and D. Hunstad, unpublished data), suggesting that DLP12 prophage-mediated bacterial lysis is not the mechanism by which YbcL is liberated from the periplasm.

Prophages in addition to DLP12 are present in both MG1655 and UTI89, although no genetic link exists between *ybcL* and these loci. Additionally, it is unclear if these prophages are competent for induction. Treatment of UTI89 pYbcL::TEM-1 with mitomycin C or hydrogen peroxide, DNA-damaging agents known to activate *recA*-dependent bacteriophage induction, during growth in RPMI alone could implicate phage induction in lysis of UTI89. Increased release of YbcL::TEM-1 under these conditions would suggest that one or more prophages are competent for induction. Ultimately, implication of a prophage in YbcL release would require mutation of the phage lysis genes and a corresponding decrease in YbcL::TEM-1 levels in the supernatant during infection of bladder epithelial cells.

The development of a β-lactamase reporter assay facilitated precise investigation into YbcL release from the periplasm. Assuming the dynamics of YbcL::TEM-1 release mimic the dynamics of release of the wild-type YbcL variant, we conclude that release of YbcL from the periplasm occurs in a dose-and time-dependent manner. In addition, the presence of YbcL in the supernatant was also dependent upon cultured bladder epithelial cells. Dependency on dose, time, and bladder epithelial cells suggests that this process may be regulated, although it is currently unclear how this is achieved. These data are consistent with transcriptional data that demonstrated that *ybcL* was up-regulated during UPEC infection of cultured bladder epithelial cells and human PMN (1). Release of YbcL::TEM-1 from the periplasm has not yet been assessed in the presence of PMN. Given the transcriptional data and the findings using bladder epithelial cells, we hypothesize that release of YbcL::TEM-1 would exhibit similar dynamics in the presence of PMN.

Bacterial cytoplasmic proteins GroEL and RNA Polymerase α subunit were detected in filter sterilized, TCA-precipitated supernatant by Western blot after UPEC infection of cultured

bladder epithelial cells. However, the cytoplasmic fusion Gst::TEM-1 was not detected in the supernatant under similar conditions. In an effort to clarify these observations, additional TEM-1 fusions with cytoplasmic localization could be generated and their release into the supernatant assessed. Initially, we hypothesized that the presence of cytoplasmic proteins in the supernatant was the result of bacterial lysis. However, it is not clear how release of cytoplasmic proteins could be selective during lysis. The presence of cytoplasmic proteins in the supernatant in the absence of bacterial lysis would imply that these proteins crossed two bacterial membranes, a phenomenon unlikely to occur incidentally. Given the well-characterized roles for RNA Polymerase α subunit and GroEL in transcription and protein folding, respectively, both cytoplasmic processes, it is unlikely that these proteins would be purposefully secreted into the supernatant. It is possible that the cytoplasmic proteins in the supernatant represent contamination from the overnight bacterial culture, despite extensive washing of the bacterial inoculum. A working hypothesis is that the bacterial outer membrane increases in permeability in the presence of bladder epithelial cells, nonspecifically releasing periplasmic contents. Detection of Skp::TEM-1 in the supernatant is consistent with this hypothesis. Increased permeability of the outer membrane in a nonselective manner could be mediated by bladder epithelial cell products. Additional experiments are required to clarify these observations.

Through targeted deletion, we demonstrated that a T2SS, a T4PS, the flagella biosynthesis machinery and the DLP12 phage lysis genes are not responsible for release of YbcL from the periplasm during UPEC infection. In light of these findings, one hypothesis is that release of YbcL is mediated by other UPEC-encoded proteins, possibly via bacterial lysis. To take a more general approach to identify potential bacterial proteins involved in YbcL liberation, we could conduct a transposon mutant screen using the β-lactamase reporter assay. Compared to

probing filter sterilized, TCA-precipitated supernatant by Western blot, the β-lactamase reporter assay is amenable to high-throughput experimentation. YbcL is transcriptionally up-regulated and then released into the supernatant during UPEC infection of cultured bladder epithelial cells (1). Potential bacterial machinery responsible for YbcL release may be similarly regulated. In addition to the transposon mutant screen, we could reexamine the data generated from the transcriptional profiling experiment that identified YbcL, looking for genes that could mediate YbcL release.

In contrast to other bacterial pathogens, the number of proteins shown thus far to be secreted by UPEC is low, and, consequently, our understanding of UPEC secretion is rudimentary. Two secreted effectors, α-hemolysin and Cnf1 are secreted by a T1SS and OMVs, respectively (9, 21). Though T2SS and T4PS have been implicated in UPEC pathogenesis (10), substrates of these systems have not been identified. Given the lack of involvement of traditional secretion systems, the mode of release of YbcL from the periplasm may represent a novel mechanism of bacterial secretion. Alternatively, liberation of YbcL may be mediated by eukaryotic products rather than bacterial products. Additional experimentation is required to address these hypotheses.

Suppression of Transuroepithelial PMN Migration by YbcL

UPEC YbcL suppresses PMN migration across a bladder epithelium in an *in vitro* model of transepithelial PMN migration and an *in vivo* murine model of cystitis (1). The increased PMN migration observed in the absence of YbcL compared to in the presence of YbcL was not the result of increased chemokine production, increased epithelial permeability, or increased

PMN mobility. Though hypotheses have been rejected, the mechanism by which YbcL suppresses PMN migration is still unclear.

During UPEC infection, YbcL was detected by Western blot in both bladder epithelial cell and PMN lysates (1). Additionally, YbcL suppressed PMN migration elicited by fMLF in the presence of bladder epithelial cells on the Transwell inserts, but had no effect in the absence of bladder epithelial cells. These data might suggest that the site of action of YbcL is the bladder epithelium rather than the PMN, despite detection of YbcL in PMN lysate. Previously, we hypothesized that the YbcL signal in the PMN lysate originated from bacteria that had been phagocytosed and killed. To test this hypothesis, we attempted to restrict YbcL to one cell type in the transepithelial PMN migration assay. Bladder epithelial cells on Transwell inserts were preincubated with purified YbcL before the epithelial cells were washed and the level of PMN migration in response to fMLF was assessed. In a similar experiment, PMN, rather than bladder epithelial cells, were preincubated with purified YbcL. In both cases, YbcL reduced the level of PMN migration (M. Lau and D. Hunstad, unpublished data). It is likely that removal of excess YbcL was incomplete. Given the low concentration of YbcL required to suppress PMN migration (<10 pM) (1), it is not surprising that reduced PMN migration was observed upon pre-exposure of either cell type if extracellular YbcL was still present.

An alternative approach to restrict YbcL localization to one cell type is ectopic expression. While cultured bladder epithelial cells are amenable to transfection and transduction, human PMN are not due to the short half-life observed after harvest from the bloodstream. However, we could evaluate the ability of HL-60 cells, a cell line generated from human promyelocytic leukemia cells which can be differentiated chemically into neutrophil-like cells, to migrate across a cultured bladder epithelium in response to UPEC infection and peptide

chemoattractants (22). In contrast to PMN, HL-60 cells can be transfected. If HL-60 cells exhibit phenotypes similar to human PMN in the transepithelial PMN migration assay, then these cells would enable a number of additional experimental approaches that are unfeasible with human PMN. If expression of YbcL in cultured bladder epithelial cells or HL-60 cells does not result in low levels of PMN or HL-60 migration in the transepithelial migration assay, then YbcL may function from the extracellular milieu.

In another approach to determine the site of action of YbcL, we developed a protocol for imaging bladder epithelial cells and PMN on Transwell inserts by confocal fluorescence microscopy. Our attempts to visualize YbcL localization in the transepithelial PMN migration assay, where YbcL has been shown to have an effect, were unsuccessful. Again, the level of YbcL required to suppress PMN migration may have been too low to detect by microscopy, especially if the localization pattern was diffuse. Alternatively, YbcL may function primarily from an extracellular location, which would require a different protocol for visualization.

As mentioned previously, YbcL was detected in bladder epithelial cell and PMN lysates during UPEC infection (1). Given the protocol used to generate cell lysate, it is impossible to distinguish between YbcL localization to the cellular membrane and YbcL localization to the cytoplasm. Fractionation of infected cells could be conducted to distinguish between these possibilities. It is unclear how YbcL might achieve cytoplasmic localization, although it may depend in part on bacterial invasion. These uncertainties could be addressed using purified YbcL. In addition to elucidating YbcL localization at the subcellular level, these experiments would inform protein-protein interaction studies, as interaction between YbcL and a membrane protein might be best detected using alternative protocols such as Far Western blot.

Traversal of an epithelial barrier by PMN is a multistep process that requires the coordinated activity of multiple adhesins and signaling pathways (23, 24). Initially, PMN adhere to the basolateral face of the epithelium. Then, they move between epithelial cells, causing the rearrangement of cell-cell contacts without disrupting epithelial integrity. After transit through tight junctions, PMN adhere to the apical face of the epithelial and are released into the lumen. It is currently unclear at which step in this complex pathway UPEC inhibit PMN migration. In the transepithelial PMN migration assay, the number of PMN in the lower reservoir represents PMN released into the lumen. As the apical sides of the Transwell inserts are lightly scraped during collection of PMN, it is unlikely that UPEC-mediated inhibition of PMN migration occurs by preventing detachment of PMN from the apical face of the epithelium. The concentration of PMN remaining in the upper reservoir is too low to reliably determine by hemacytometer. To assess PMN levels in the upper reservoir and PMN associated with the bladder epithelium, a more sensitive method for detecting PMN is required. An assay based on myeloperoxidase (MPO) activity or MPO protein levels may enable more accurate quantitation of PMN. Additionally, PMN association with the bladder epithelium could be investigated using confocal fluorescence microscopy. Given the amorphous appearance of the epithelium, this endeavor may be difficult. Understanding the point at which UPEC inhibit PMN migration may highlight adhesins or signaling pathways that could be subject to inhibition by YbcL.

To further interrogate how YbcL suppresses PMN migration, understanding the molecular requirements for traversal of a bladder epithelial barrier by PMN is required. Significant work has been done in the gastrointestinal tract and lung to identify the adhesins required for PMN to transit these epithelial barriers (23, 25). It is unclear if the same adhesins play a role in the bladder. Agace and colleagues have demonstrated that expression of

intercellular adhesion molecule-1 (ICAM-1) by the bladder epithelial cell line J82 and expression of the β–integrin CD11b/CD18 (Mac-1) by PMN is required for traversal of the epithelial layer in their model (26). The involvement of other adhesins (e.g., SIRPα, CD47, CAR) that have been implicated at other epithelial surfaces has not yet been examined. Expression of these adhesins by cultured bladder epithelial cells and PMN could be validated by quantitative PCR and Western blot. The requirement of these proteins for PMN migration in the transepithelial PMN migration assay could be examined using blocking antibodies. These findings could be extended to the murine model of cystitis through the use of genetically modified mice.

The movement of PMN across epithelial barriers is a highly complex process that can be influenced by many factors. We demonstrated that the differential PMN migration observed upon infection with wild-type UTI89 and UTI89 *ybcL::cat* in the transepithelial PMN migration assay was not the result of differences in chemoattractant gradients, epithelial integrity, or PMN mobility. The challenge moving forward is that the current understanding of PMN recruitment to the bladder is incomplete. To interrogate a role for YbcL, there is a need to investigate the basic processes required for PMN migration at this site, focusing on adhesion interactions and signaling cascades. Findings at other epithelial surfaces will inform these studies.

Concluding Remarks

In summary, the goal of this thesis work was to contribute to the current understanding of host-pathogen interactions in the urinary tract. UPEC employ multiple strategies to suppress and delay the acute inflammatory response in the bladder, extending the period during which UPEC can initiate an intracellular reservoir in the absence of immune pressure. As formation of this intracellular reservoir is required to propagate infection, the events preceding bacterial invasion

have the potential to influence disease outcome. This study identified a UPEC protein YbcL that contributes to modulation of the innate immune response by suppressing the migration of neutrophils across bladder epithelia. YbcL is the first UPEC protein implicated in this phenotype. Additionally, this work demonstrated that YbcL was released from the periplasm, though this liberation was not mediated by a canonical secretion system. YbcL represents the first UPEC protein shown to function outside the bacterial periplasm without an identified mode of release. Finally, the mechanism by which YbcL influences PMN migration appears to be unique, as hypotheses based on the functions of other bacterial effectors were disproved. Although additional experimentation is required to clarify some of these findings, this thesis extends our understanding of strategies used by uropathogens to manipulate the innate immune response during infection.

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APPENDIX I

QUANTITATIVE ASSESSMENT OF HUMAN NEUTROPHIL MIGRATION ACROSS A CULTURED BLADDER EPITHELIUM

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Modified from Lau and Hunstad (2012) *J Vis Exp.* In press.

Short Abstract

We developed an *in vitro* model that mimics an important component of the acute inflammatory response during infection of the bladder with uropathogenic *Escherichia coli.* The transuroepithelial neutrophil migration assay enables quantitative assessment of human neutrophil migration across bladder epithelia, cultured on permeable supports, in response to bacterial infection or chemoattractant substances.

Long Abstract

The recruitment of immune cells from the periphery to the site of inflammation is an essential step in the innate immune response at any mucosal surface. During infection of the urinary bladder, polymorphonuclear leukocytes (PMN; neutrophils) migrate from the bloodstream and traverse the bladder epithelium. Failure to resolve infection in the absence of a neutrophilic response demonstrates the importance of PMN in bladder defense. To facilitate colonization of the bladder epithelium, uropathogenic *Escherichia coli* (UPEC), the causative agent of the majority of urinary tract infections (UTIs), dampen the acute inflammatory response using a variety of partially defined mechanisms. To further investigate the interplay between host and bacterial pathogen, we developed an *in vitro* model of this aspect of the innate immune response to UPEC. In the transuroepithelial neutrophil migration assay, a variation on the Boyden chamber, cultured bladder epithelial cells are grown to confluence on the underside of a permeable support. PMN are isolated from human venous blood and are applied to the basolateral side of the bladder epithelial cell layers. PMN migration representing the physiologically relevant basolateral-to-apical direction in response to bacterial infection or chemoattractant molecules is enumerated using a hemacytometer. This model can be used to investigate interactions between UPEC and eukaryotic cells as well as to interrogate the molecular requirements for the traversal of bladder epithelia by PMN. The transuroepithelial neutrophil migration model will further our understanding of the initial inflammatory response to UPEC in the bladder.

Introduction

The movement of cells throughout the body, often across long distances, is required for growth and development, wound healing, and immune response. Cell migration is complex and requires the coordination of many different processes, including signaling cascades and the

rearrangement of cytoskeletal components. Cells can move randomly (chemokinesis) as well as toward defined chemical gradients (chemotaxis). Many techniques have been developed to study cell migration *in vitro*. The oldest and most common technique, the Boyden chamber, consists of a vertical two-chamber system where a chemoattractant substance is placed in the bottom chamber and cells of interest are placed in the top chamber (1) . The movement of cells across the permeable filter, with pores of defined size, separating the two chambers is monitored. Additional techniques have been developed to investigate cell migration including the Zigmond chamber (2) and the Dunn chamber (3). These collective approaches have yielded significant insight into the movement of many different cell types.

In addition to interrogating the basic principles of chemokinesis and chemotaxis, two-chamber assays have facilitated the investigation of cell migration through extracellular matrix components and both endothelial and epithelial cell layers. An advantage of two-chamber systems over other techniques is that the porous membrane can be coated with proteins such as collagen or fibrinogen, and cell migration across an extracellular matrix-like barrier can be assessed. Additionally, cultured cell lines can be grown and differentiated on the permeable supports. To investigate the movement of cells across an endothelial barrier, cultured endothelial cells are seeded and grown in the upper reservoir of the permeable supports. Motile cells, such as immune cells, are added to the upper reservoir and migration into the lower reservoir across the endothelial barrier in the physiologic apical-to-basolateral direction is observed. This model has been invaluable in understanding extravasation of immune cells out of the blood stream. In contrast to transendothelial migration, the movement of cells across an epithelial barrier typically occurs in the basolateral-to-apical direction. In order to model these events *in vitro*, researchers seed and grow cultured epithelial cells on the underside of the permeable supports. Motile cells

are added to the upper reservoir and migration across the epithelial barrier, representing the basolateral-to-apical direction, is monitored. Such models of transepithelial migration have significantly contributed to our understanding of inflammatory responses at mucosal surfaces, particularly those of the lung and gut (4, 5).

In contrast, immune cell trafficking through the epithelium of the urinary tract has received much less attention. To further our understanding of innate immune responses in the urinary tract during infection with uropathogenic *Escherichia coli* (UPEC), we developed an *in vitro* assay, the transuroepithelial neutrophil migration assay, that enables investigation of polymorphonuclear leukocyte (PMN; neutrophil) movement across a bladder epithelial barrier (6-8). As with other two-chamber models of transepithelial migration, cultured human bladder epithelial cells are grown on the underside of a permeable support and form confluent epithelial layers. Human neutrophils, isolated from venous blood, are applied to the basolateral side of the epithelial layer, and migration across the epithelium in the physiologically relevant basolateral-to-apical direction is quantified in response to infection with different strains of *E. coli* or the presence of chemoattractant molecules. Much research had focused on neutrophil movement, both chemokinesis and chemotaxis, in the absence of additional cell types. The transuroepithelial neutrophil migration assay is advantageous as it takes into account complex interactions between bladder epithelial cells and immune cells during infection. This tractable *in vitro* model has the potential to permit the detailed investigation of immune responses at uroepithelial surfaces.

Protocol

1) Culturing 5637 Bladder Epithelial Cells

Perform the following steps in a laminar flow tissue-culture hood prepared with UV irradiation and wiped down with 70% ethanol.

1.1) Prepare RPMI-1640 medium containing 10% fetal bovine serum (FBS) [termed RPMI+], filter sterilize using a 0.22-µm pore size filter, and warm to 37°C in a water bath.

1.2) Thaw a cryovial of 5637 cells (American Type Culture Collection HTB-9; derived from bladder carcinoma) in a 37°C water bath. Quickly transfer the thawed cells to a 75 cm^2 tissue culture flask containing 20 ml RPMI+.

1.3) Incubate the flask at 37°C in a humidified atmosphere with 5% CO_2 until the cells are approximately 95% confluent (~6×10^6 cells per flask), about 4 days.

1.4) To subculture the 5637 cells:

1.4.1) Remove the medium from the flask. Wash the cells with 10 ml Dulbecco's Phosphate-Buffered Saline (DPBS) at room temperature.

1.4.2) Add 6 ml warm (37°C) 0.05% trypsin, 0.02% EDTA solution to the flask, and incubate at 37°C with 5% CO_2 for 15 min.

1.4.3) Transfer the cells to a 15 ml conical tube, and pellet by centrifugation at 300 × *g* for 5 min. Remove the trypsin/EDTA solution.

1.4.4) Resuspend the cells in 6 ml RPMI+ (10^6 cells/ml), and transfer 1 ml (10^6 cells) to a new 75 cm^2 flask containing 20 ml RPMI+.

1.5) Repeat step 1.4 to subculture cells every 4 days or when the cells reach 95% confluence.

2) Seeding and Growing 5637 Cells on Permeable Supports

Perform the following steps in a tissue culture hood using aseptic technique. For optimal results, use 5637 cells that have undergone fewer than 10 subcultures.

2.1) Using sterile forceps, invert the permeable supports in a sterile 25 mm deep tissue culture dish.

2.2) Trypsinize a 75 cm^2 flask of 5637 cells at 95% confluence according to steps 1.4.1-1.4.3.

2.3) Using a 1000 µl pipet, gently resuspend the cells in ~ 2 ml RPMI+ to a concentration of 3×10^6 cells/ml, determined by cell counting using a hemacytometer.

2.4) Apply 50 µl of the cell suspension (1.5×10^5 cells) to each permeable support without touching the membrane. Place the lid on the dish, and carefully place the dish at 37°C with 5% CO_2 for no more than 16 h.

2.5) Using sterile forceps, right the permeable supports into a 24-well plate containing 0.6 ml RPMI+ per well. Add 0.1 ml RPMI+ to the upper reservoir of each permeable support. Incubate at 37°C with 5% CO_2.

2.6) Replace the medium every 2 days. First, aspirate medium from the upper reservoir followed by the lower reservoir, and then apply fresh RPMI+ in the reverse order, to the lower reservoir (0.6 ml) and then the upper reservoir (0.1 ml).

2.7) Seven days after seeding the permeable supports with 5637 cells, assess confluence of the cells. Fill the upper reservoir with RPMI+ (~0.35 ml). The cells are sufficiently confluent when the medium does not equilibrate between the upper and lower reservoirs.

3) Preparation of the Bacterial Inoculum

3.1) Using aseptic technique, add 20 ml of appropriate culture broth to a 250 ml flask with a cap. Use Luria-Bertani (LB) broth for *E. coli* cultures, and add antibiotics to the broth where appropriate.

3.2) Using a sterile inoculation loop, inoculate the broth with bacteria from a glycerol stock or a streak plate. For UPEC strains, we incubate the bacterial culture at 37°C for approximately 16 h, without shaking (to promote production of type 1 pili).

3.3) Transfer the bacterial culture to a centrifuge tube. Pellet the bacteria by centrifugation at 8000 × *g* for 10 min.

3.4) Decant the supernatant, and resuspend the bacteria in PBS to $OD_{600\ nm}$ = 1, equivalent to ~10^9 CFU/ml.

3.4.1) To generate a heat-killed bacterial stimulus, incubate an aliquot (e.g., ~1.5 × 10^8 CFU in 150 μl) of the resuspended bacteria at 55°C for 30 min. Plate an aliquot of the heat-killed suspension to confirm bacterial death.

3.5) Immediately before use, dilute the resuspended bacteria (live or heat-killed) 10-fold to 10^8 CFU/ml in warm serum-free RPMI [termed RPMI-] in a microfuge tube.

4) Isolation of Human Neutrophils from Peripheral Blood

The collection of blood from adult volunteers requires advance review and approval from an institutional review board. Wear appropriate personal protective equipment and properly dispose of hazardous materials to avoid exposure to human blood.

4.1) Approximately 25 ml of venous blood from a healthy adult volunteer should be drawn into 3 sterile sodium heparin-containing blood collection tubes by staff trained in phlebotomy.

4.1.1) Tightly wrap a rubber tourniquet around the upper arm, above the elbow.

4.1.2) Disinfect the entry site, the antecubital fossa, using a sterile 70% alcohol wipe.

4.1.3) Attach a plastic tube holder to a winged butterfly needle system.

4.1.4) Insert the needle into an antecubital vein at a 30° angle or less, bevel facing up. The needle has punctured the vein when a spurt of blood appears in the plastic tubing.

4.1.5) Insert a blood collection tube into the plastic tube holder. When the first collection tube is full, replace with the second collection tube. Repeat with the third tube.

4.1.6) When the third collection tube is approximately half full, remove the tourniquet.

4.1.7) Cover the puncture site with a sterile cotton ball and slowly withdraw the needle from the vein. Slide the protective shield over the needle and place in a biohazard sharps container.

4.1.8) Apply pressure to the puncture site. Cover the puncture site and cotton ball with an adhesive bandage.

4.1.9) Gently invert the blood collection tubes to disperse the heparin.

Perform the following steps in a tissue culture hood using aseptic technique.

4.2) Using a 10 ml pipet, gently transfer the blood to a fresh, sterile 50-ml conical tube. Add an equivalent volume of 3% (w/v) dextran in 0.9% NaCl and mix by inversion. Incubate the tube upright at room temperature for 20 min.

4.3) Without disrupting the lower layer, carefully aspirate the upper layer and transfer it to a new 50 ml conical tube. Pellet the cells by centrifugation at 300 × g for 10 min. Discard the supernatant.

4.4) Resuspend the cell pellet in a volume of 0.9% NaCl equivalent to the starting volume of blood.

4.5) Layer 10 ml of density centrifugation solution under the cell suspension, preserving the interface between the two phases. Centrifuge at 400 × g for 30 min with no brake. Discard the supernatant.

4.6) To lyse remaining red blood cells, resuspend the cell pellet in 10 ml cold 0.2% NaCl. Incubate for 30 sec, and then promptly add 10 ml cold 1.6% NaCl to restore isotonicity.

4.7) Pellet the cells by centrifugation at 300 × *g* for 6 min. Discard the supernatant.

4.8) Repeat steps 4.6-4.7 until the cell pellet appears to be free of red blood cells, typically 3 rounds of lysis.

4.9) Using a 1000 µl pipet, resuspend the cell pellet, primarily PMN, in warm (37°C) RPMI- to a concentration of 10^7 cells/ml, determined by cell counting using a hemacytometer. Keep the cells at 37°C until use. Typically, PMN viability and purity are > 99% as assessed by trypan blue exclusion and visualization of nuclear morphology after staining, respectively.

5) Transuroepithelial Neutrophil Migration Assay

Perform the following steps in a tissue culture hood using aseptic technique. Use only permeable supports bearing confluent 5637 cell layers, as determined in step 2.7. The 24-well plates containing RPMI- can be prepared in advance and kept at 37°C with 5% CO_2 until use.

5.1) Aliquot 1 ml warm RPMI- per well in a 24-well plate; prepare 3 wells per permeable support.

5.2) Aspirate the medium from the upper and lower reservoirs of the permeable supports.

5.3) Using sterile forceps, transfer the permeable supports to the 24-well plate prepared in step 5.1. Wash the permeable supports three times by transferring the supports from well to well.

5.4) If a bacterial inoculum is to be used, invert the permeable supports in a sterile 25 mm deep tissue culture dish. If a chemoattractant (e.g., N-Formyl-Met-Leu-Phe (fMLF) or IL-8) is to be used, proceed to step 5.6.

5.5) For experiments with live or killed bacterial stimuli, inoculate the apical side of each permeable support with 60 µl RPMI- (mock infection) or with the bacterial inoculum (6×10^6 CFU) prepared in step 3.5. Place the lid on the dish and incubate at 37°C with 5% CO_2 for 1 h.

5.6) Add 0.6 ml RPMI- per well to a 24-well low-attachment plate; prepare 1 well per permeable support. Prepare 3 wells containing 0.5 ml RPMI- to enumerate PMN input.

5.6.1) If a chemoattractant is being used in place of bacteria, add the chemoattractant to 0.6 ml RPMI- in the 24-well plate prepared in step 5.6.

5.7) Right the permeable supports into the 24-well low-attachment plate.

5.8) Add 0.1 ml PMN (10^6 PMN), prepared in step 4.9, to the upper reservoir (basolateral side) of each permeable support. Add 0.1 ml PMN directly to wells containing 0.5 ml RPMI-. Incubate at 37°C with 5% CO_2 for 1 h.

5.9) Using sterile forceps, gently scrape the membrane of the permeable support against the edge of the well to remove additional PMN from the apical side and then dispose of the support.

5.10) Collect PMN by gently scraping the bottom of each well with the 1000-µl pipet tip, and transfer PMN suspensions to microfuge tubes.

5.11) Enumerate PMN using a hemacytometer.

5.12) To calculate the total number of PMN in the lower reservoir, multiply the number of PMN in 1 mm^2 (100 nl) by 6000. Data can be reported as a proportion of input PMN that have migrated, or as absolute numbers of PMN.

Representative Results

The transuroepithelial neutrophil migration assay enables the quantitative assessment of human PMN migration across cultured bladder epithelial cell layers in response to various

stimuli **(Figure 1B)**. While the protocol is straightforward, there are a number of variables that can influence PMN migration and consequently affect the reproducibility of this assay. Measures should be taken while preparing the permeable supports and the PMN to reduce variability between technical and biological replicates. For example, only permeable supports containing sufficiently confluent 5637 cell layers should be used in an experiment. Confluence of the 5637 cells is assessed using a functional assay that measures impermeability to liquid. If medium added to the upper reservoir equilibrates across the permeable support, then the 5637 cells are not sufficiently confluent to conduct the experiment. If the volume in the upper reservoir is maintained, then the permeable support can be used to assess PMN migration. We have measured transepithelial electrical resistance in this system, which rises modestly upon confluence of the cells; if this method is chosen, care should be taken not to contaminate the otherwise sterile setup. Confluence of the 5637 cells 7 days after seeding can be influenced by multiple factors, including the passage number of the cells and the number of cells seeded on the permeable support. In addition, the amount of time that the 5637 cells are incubated on the permeable support in the inverted position during seeding should not exceed 16 h **(Figure 1A)**. For optimal reproducibility, the protocol should be followed precisely. Finally, permeable supports containing confluent 5637 cell layers should be used within 1-2 days, and the membranes of the supports should never be touched during either growth of the 5637 cells or during the transuroepithelial neutrophil migration assay.

In addition to the 5637 cells, variability can also be introduced during PMN preparation. Using the protocol detailed above to isolate PMN, 1 ml of human blood typically yields about 10^6 PMN, although this number varies from individual to individual. Once the typical yield of an individual donor's blood is known, the isolation protocol can be scaled up or down

accordingly. PMN from unhealthy or ill individuals should be avoided, and different PMN donors should be used for biological replicates to ensure that results observed are reproducible. PMN should be handled gently and sterilely to avoid activation during isolation. Lastly, the timing of the experimental procedures is crucial, as PMN do not survive for extended periods of time once removed from the body. We utilize PMN within 1 h of completing the isolation procedure. Given these considerations, at least 3 technical replicates should be included in each biological replicate.

The number of PMN in the lower reservoir after 1 h is shown in **Figure 2** normalized to 10^6 input PMN. Alternatively, PMN numbers can be compared to an internal control after normalization to input PMN, which may reduce variation between biological replicates. Adherence to the protocol outlined above with attention to detail enables the enumeration of PMN migration in response to stimuli including bacteria **(Figure 2A)** and chemoattractant substances **(Figure 2B)**.

Discussion

Using a cultured bladder epithelial cell line and freshly isolated human PMN, we established an *in vitro* model of transuroepithelial neutrophil migration. This model has been instrumental in beginning to dissect the complexities of the innate immune response during urinary tract infection (UTI), an extremely common bacterial infection typically caused by UPEC (9). During infection of the bladder, or cystitis, recruitment of PMN to the bladder lumen is essential for bacterial clearance (10). To establish a foothold in the face of an inflammatory response, UPEC delay the arrival of PMN to the bladder, which prolongs the period during which UPEC can invade the bladder epithelium in the absence of immune pressure (6, 11). This

phenotype, suppression of PMN migration by UPEC at early time points, is observed in our *in vitro* model of transuroepithelial PMN migration. Infection with nonpathogenic *E. coli* MG1655 elicits significantly more PMN migration than infection with uropathogenic *E. coli* UTI89 (**Figure 2A**); co-infection with MG1655 and UTI89 yields the uropathogenic phenotype (i.e., low levels of PMN migration) (6). Furthermore, we identified a UPEC protein, YbcL, that contributes to the suppressive phenotype, as deletion of *ybcL* resulted in significantly more PMN in the lower reservoir compared to wild-type UTI89 (**Figure 2A**) (7). High levels of PMN migration were also elicited by heat-killed MG1655, fMLF and IL-8 (**Figure 2A and B**). In comparison to infection with a live bacterial stimulus, the use of chemoattractants may simplify both the experimental protocol and the interpretation of results. It is likely that other chemoattractants (e.g., bacterial products or chemokines) would also elicit PMN in this model. Thus far, the transuroepithelial neutrophil migration assay has facilitated investigations into suppression of the early inflammatory response by UPEC, revealing phenotypes that have been verified in *in vivo* models (6-8), and will be an invaluable tool in future endeavors.

While this assay has the potential to address a number of questions, there are a few limitations. Given the number of variables inherent to this assay, care must be taken while preparing and conducting experiments to ensure reproducibility between replicates. Enumerating PMN by counting is prone to error, as counting can be time intensive and PMN are short-lived once removed from the body, especially in the presence of bacteria. Reducing the time between PMN sample collection and PMN enumeration will result in more accurate data collection. Researchers have described colorimetric assays that measure myeloperoxidase (MPO) enzyme activity as a surrogate for PMN (12, 13). Assays utilizing colorimetric substrates such as 3, 3', 5, 5',-tetramethylbenzidine (TMB) or 2, 2'-azino-bis (3-ethylbenzothiazoline-6-

sulphonic acid) (ABTS) are not sufficiently sensitive to detect the concentrations of PMN present in the lower reservoirs using the transuroepithelial neutrophil migration protocol detailed above (Lau and Hunstad, unpublished data). The parameters of the migration assay could be manipulated to increase the PMN density in the lower reservoir samples. Alternatively, an MPO assay with greater sensitivity, potentially utilizing a fluorescent substrate, could be established. Measurement of MPO activity represents an alternative to PMN counting and may enable more accurate enumeration of PMN in the lower reservoir samples. Additionally, such an assay may also allow enumeration of PMN adherent to the epithelial layers and remaining in the upper reservoir. A validated MPO-based protocol would represent a powerful tool that could expand the amount and type of data that could be collected from the transuroepithelial neutrophil migration assay.

Transepithelial neutrophil migration assays modeling the innate immune response in the gastrointestinal tract and lung are widespread and are responsible for our current understanding of the traversal of epithelial barriers by PMN (4, 5). In contrast, PMN movement across uroepithelial barriers has received far less attention. Säve and colleagues have reported a model that uses a polarized epithelium composed of differentiated UROtsa cells, an immortalized cell line derived from ureter tissue, grown to confluence on permeable supports (14). Agace and colleagues have reported the use of undifferentiated bladder (J82) and kidney (A498) epithelial cells in a similar model (15). In the transuroepithelial neutrophil migration model detailed herein, though the 5637 cell layers are not stratified and likely not formally polarized, tight junctions are formed, assessed by impermeability to macromolecular flux and the expression and localization of tight junction proteins (Lau and Hunstad, unpublished data). Of note, the epithelial layer also maintains such impermeability during the infection conditions we have

described. The protocols reported by Säve and Agace model the inflammatory response after infection with UPEC isolates for 24 h. In these models, UPEC elicit robust PMN migration. In contrast, the epithelial layers in our model are exposed to UPEC for a relatively short period of time, 1 h, in order to examine the initial interactions between host and pathogen. Furthermore, the use of a bladder epithelial cell line and a cystitis-derived UPEC isolate enables potential translation of *in vitro* findings to the murine cystitis model (16). Lastly, the studies mentioned above utilized large permeable supports that require 6-well tissue culture dishes. The use of smaller permeable supports, as in our model, reduces reagent use and increases the number of inserts that can be manipulated per experiment. Although each of these model systems has advantages and disadvantages, the collective potential of these models to define events required for PMN migration into urinary tract tissues is substantial.

Although less well understood than migration across endothelial barriers, the passage of PMN across gastrointestinal and pulmonary epithelial barriers has received much study (4, 5). Transepithelial neutrophil migration models that employ permeable supports and cultured epithelial cells have revealed some of the signaling events and adhesion molecules involved in the movement of PMN through these epithelia. Preliminary studies using cultured urinary epithelial cells suggest the involvement of intercellular adhesion molecule-1 (ICAM-1) and the β–integrin CD11b/CD18 (Mac-1) in PMN migration across urinary tissues (15). It is unclear which additional signaling pathways and adhesive molecules are involved in these complex processes in the urinary tract. Using the transuroepithelial neutrophil migration model described herein, perhaps augmented with microscopic examination, these basic questions and many others can be interrogated. Additionally, in conjunction with bacterial genetics, this model can be used to further evaluate pathogen-specific phenotypes such as suppression of PMN migration by

UPEC. It is unclear at which point in the multi-step process of PMN migration UPEC exerts its suppressive effect. Also, the mechanism by which YbcL influences PMN migration has yet to be elucidated. By first understanding the basic requirements for the passage of PMN across the bladder epithelium, we can then begin to probe how UPEC manipulates these processes to facilitate disease.

In summary, while numerous techniques exist to study the movement of cells, fewer approaches are available to interrogate cell migration across cellular barriers. Modifications to the Boyden chamber have been integral to investigating cell migration across endothelial and epithelial barriers. A tractable *in vitro* model of the acute inflammatory response in the urinary tract, such as the transuroepithelial neutrophil migration assay detailed herein, is a valuable tool for interrogating these complex processes. Lastly, modifications to this assay could facilitate investigations into other disease states of the urinary tract.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants R01-DK080752 and P50-DK064540. We thank J. Loughman for her efforts in establishing this assay.

Disclosures

The authors have nothing to disclose.

Tables and Figures

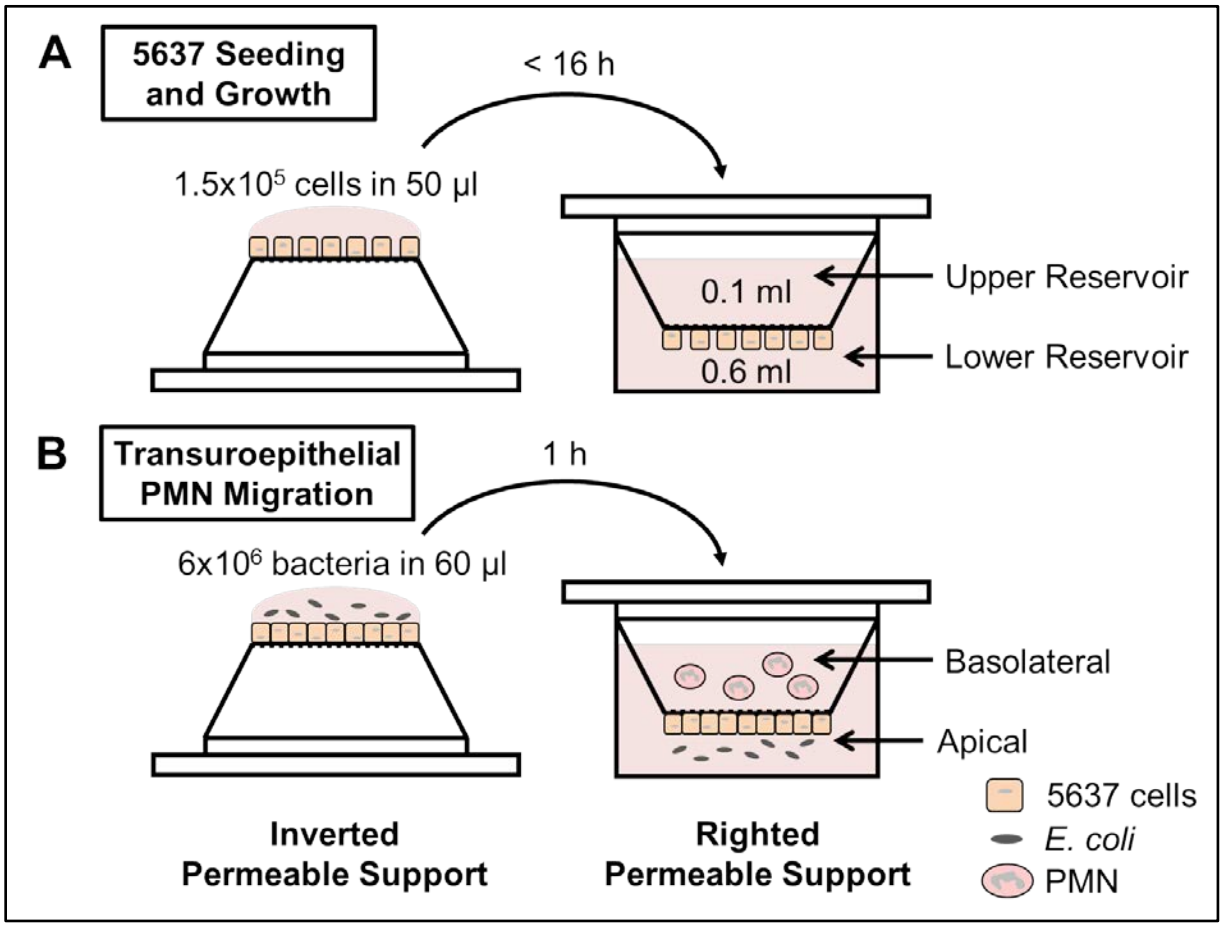

Figure 1: Schematic of experimental design. (A) 5637 bladder epithelial cells are seeded on inverted permeable supports, the supports are righted into a 24-well plate, and the cells are grown to confluence. (B) Permeable supports containing confluent 5637 cells are inverted and infected with *E. coli* on the apical side of the epithelial layers. Alternatively, chemoattractants can be placed in the lower reservoir. The permeable supports are righted into a low-attachment plate, and freshly isolated human PMN are applied to the upper reservoir (representing the basolateral side of the epithelial layers). PMN migrate across the epithelium and are enumerated from the lower reservoir using a hemacytometer.

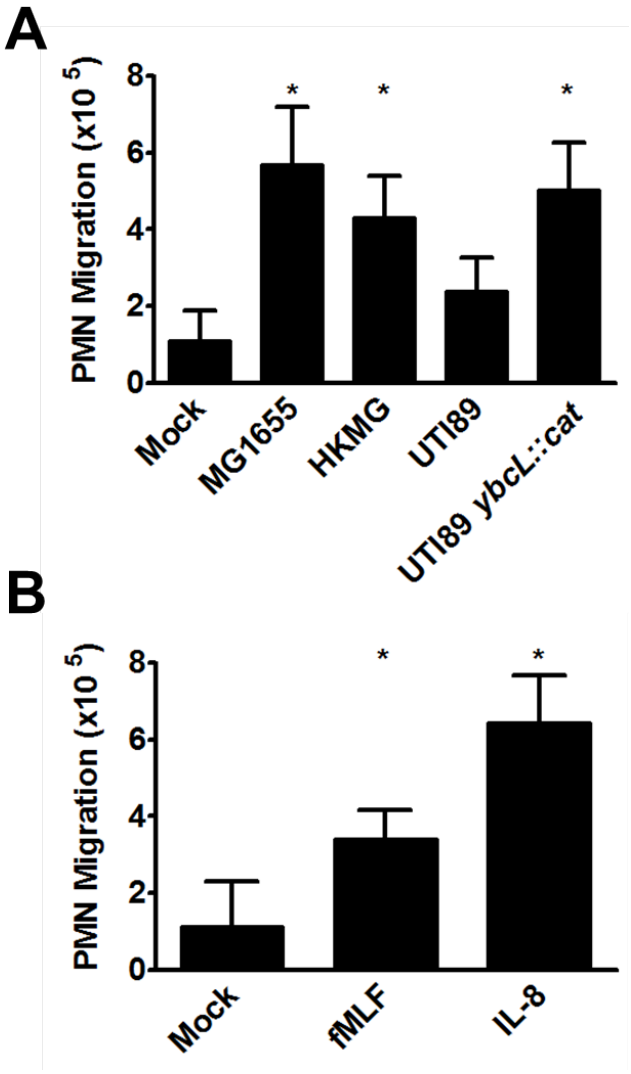

Figure 2: PMN migrate across bladder epithelia in response to various stimuli. (A) Infection with nonpathogenic *E. coli* strain MG1655, heat-killed MG1655 (HKMG) or UPEC mutant UTI89 *ybcL::cat* elicits significantly more PMN migration than mock infection or infection with wild-type UPEC strain UTI89, a cystitis isolate (* $p < 0.001$). (B) The addition of fMLF (100 nM) or IL-8 (100 ng/ml) to the lower reservoir results in significantly more PMN migration than mock treatment (* $p < 0.001$). Data represent the mean and standard deviation from at least 3 biological replicates. Statistically significant differences were determined using an unpaired Student's *t* test.

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