# THE EFFECT OF SUBSTRATE STIFFNESS ON UROPATHOGENIC *E. COLI* INFECTION

By

JULIA T. KEKLAK

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Written under the direction of

Eric A. Klein

And approved by

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Dr. Eric Klein

_____

Dr. Nir Yakoby

_____

Dr. Jongmin Nam

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THESIS ABSTRACT

The Effect of Substrate Stiffness on Uropathogenic E. coli Infection

By JULIA KEKLAK

Thesis Director:
Eric A. Klein

Both mammalian and bacterial cells respond to environmental cues and then elicit various physiological responses. Mechanical cues, such as surface attachment, have been noted as a critical part of bacterial infection through interaction with mechanosensors and the actin cytoskeleton. Urinary tract infections are a sufficient model for studying attachment due to the mechanism of bacterial infection. The cytoskeleton mediates various cell processes that are involved with infection, such as endocytosis and vesicle trafficking. The disruption of the host actin cytoskeleton leads to increased uropathogenic *E. coli* infection (UPEC). Since substrate stiffness mediates changes in host cell physiology through cytoskeletal interaction, we explored the UPEC infection process on polyacrylamide gels of differing stiffnesses. We found that UPEC escapes endocytic vesicles and replicates within the cytoplasm on soft gels, yet are trapped within lysosomal-associated membrane protein (LAMP-1) vesicles on stiff surfaces. Cytoplasmic replication is consistent with previous *in vivo* studies. Increased infection

was also found on soft gels when compared with stiff substrates. These findings may provide a more accurate cell culturing model for future UPEC infection studies.

## KEY TERMS

UPEC: Uropathogenic E. coli

UTI: Urinary tract infection

IBC: Intracellular bacterial communities

QIR: Quiescent intracellular reservoirs

LAMP-1: Lysosomal-associated membrane protein 1

5637: Human uroepithelial cell line

UTI89: clinical isolate of UPEC

## Introduction:

### Urinary Tract Infections as a Model System:

Urinary tract infections are the most common community-acquired bacterial infection and about 80% are caused by uropathogenic *E. coli* (UPEC) (Hooton and Stamm 1997). UPEC must be attachment to the bladder epithelial lining in order to successfully invade. The physical environment of the bladder is characterized by low pH and the presence of urea, organic acids, and salts that aim to deter bacterial colonization. The constant flow of urine is able to wash away nonattached or weakly attached UPEC. The uroepithelial lining also contains a mucosal layer as well as the ability to exfoliate and subsequently excrete the infected bladder umbrella cells (Mysorekar and Hultgren 2006). This process is dependent on the attachment of UPEC surface adhesions to the epithelial lining of the bladder and involves an apoptosis-like pathway.

The surface adhesion molecules that allow for attachment to the host epithelial lining are thought to be the most important determinant of pathogenicity in UPEC. The adhesins allow for the initiation of infection through binding directly to the host cell and avoiding rapid clearance with the bulk flow of urine. These adhesive organelles include type 1, P, and S/F1C-related pili and the Dr family, with type 1 pili being the most critical pilus for infection in the bladder. Type 1 pili consist of a 7 nm thick helical rod made of repeated FimA subunits. The tip contains two adaptor proteins (FimF and FimG) that connect the helical rod to the adhesin, FimH. The N-terminus of FimH is the adhesion domain that contains a carbohydrate binding pocket. The N-terminus is able to attach to the mannosylated uroplakin receptors on the surface of the uroepithelial cell, while the C-terminus is bound to the adaptor protein (Mulvey 2002). Previous studies suggest that the

adaptor proteins aid in initiating pilus assembly and act as inhibitors of pilus polymerization (Russell and Orndorff 1992). This assembly mechanism, known as the chaperone/usher pathway, is widely conserved throughout pilus-expressing bacteria. FimD is the outer membrane assembly platform and maintains a variety of important functions. It anchors the pilus to the cell membrane and is able to specifically recognize various FimC subunit complexes (Nishiyama et al. 2003). FimC is a chaperone protein that has two important functions that result in the assembly of type 1 pili. First, they facilitate the folding of FimA subunits into the correct conformation as they emerge from the cytoplasm. Second, they form soluble chaperone-subunit complexes to stabilize FimA within the periplasm, as well as preventing unwanted interactions at the exposed active sites (Schilling et al. 2001). The role of the FimH N terminal in extracellular signaling to the cytoplasm has remained unclear.

Figure 1: The assembly of type 1 pili begins within the UPEC periplasm.

Type 1 pili are vital to bacterial uptake and subsequent changes within the host actin cytoskeleton. SEM and TEM studies have noted that UPEC internalization triggered by type 1 pilus involve a zipper mechanism which leads to local changes in the host cell membrane. UPEC is then able to become internalized through a cascade of signaling

events that is also mediated by the host cytoskeleton (Bien et al. 2012). Thus, it is important to characterize the relationship between bacterial attachment, internalization, and the host actin cytoskeleton (Mulvey et al. 2001).

UPEC Internalization and Exocytosis:

UPEC internalization begins with the attachment of FimH to mannosylated uroepithelial surface receptors, such as uroplakin, α3, and β1 integrins. Immediately after binding, UPEC is internalized into a cyclic AMP modulated Rab27b/CD63+ vesicle. There are several host cell components that are required for internalization such as Rho GTPases, kinases, and several other signaling intermediates. There are several types of vesicles that are responsible for endocytic transport that are marked by differing classes of Rab GTPase proteins. The maturation of early to late endosomes is characterized by a shift from Rab5 to Rab7 regulation. Rab7 activation is required for the transition from early to late endosomal formation. Late endosomes are typically targeted for lysosomal degredation (Sun et al. 2010).

Once internalized by the host cell, a majority of bacterial cells are then subsequently expelled from the host cell through Rab27b mediated exocytosis. However, some UPEC cells are able to escape the vesicle before it is expelled and begin to quickly form intracellular bacterial communities (IBCs) in the superficial umbrella cells. The Rab27b/CD63+ vesicles belong to a class of exocytic vesicles that act as secretory organelles. Secretory vesicle exocytosis is induced by intracellular calcium levels and cyclic AMP. One study noted that 85% of internalized UPEC is contained in Rab27b and CD63 associated secretory vesicles (Bishop et al. 2007). Within the immature, undifferentiated underlying epithelial cells, the Rab27b/CD63+ vesicles are entangled

within a vast network of actin fibers and thus, UPEC proliferation is limited. Interestingly, exocytosis of the UPEC-containing secretory vesicles can be enhanced by induction of host actin cytoskeletal rearrangement (Dhakal et al. 2008).

UPEC Quiescent Reservoirs and Intracellular Bacterial Communities:

Recently, studies found that UPEC are able to activate a complex cascade of molecular events upon internalization into the uroepithelial cell. The FimH adhesin is able to directly stimulate host cell signaling pathways that lead to cytoskeletal rearrangement and envelopment of attached bacterial cells (Martinez et al. 2000). Previously, it was alleged that bacterial internalization was a result of host cell immunity. However, it was recently noted that bacterial cells benefit from this internalization. In a murine model, it was found that internalized bacteria had a higher survival advantage compared with extracellular bacteria. Intracellular UPEC are able to avoid contact with host cell immune defenses and antimicrobial constituents. One study in murine bladders revealed that internalized bacteria are able to avoid contact with antibiotics that significantly reduce extracellular bacteria (Mulvey et al. 1998; Mulvey et al. 2001).

Initial invasion and establishment of IBCs persist for 1-3 hours after inoculation. They then begin to rapidly replicate within the undifferentiated cell cytoplasm (Anderson et al. 2004). However, *in vitro* studies found that UPEC remain within lysosomal-associated membrane protein 1 (LAMP-1) vesicles during this stage of infection (Berry et al. 2009). LAMP-1 is a transmembrane protein that is expressed in several human tissue types. In few cases, LAMP-1 has been found to be associated with the cell membrane and is able to mediate cell-cell adhesion through lectin binding (Acevedo-Schermerhorn et al. 1997).

During the late stages of intracellular bacterial community (IBC) formation, UPEC growth begins to slow down and they display a coccus-like morphology. At this stage, they begin to form a tightly regulated, community-like aggregate that is thought to enhance resistance to host immunity. Bacterial cells located on the edges of this aggregate dislodge from the group and return to its original rod-shaped morphology. They eventually exit the host cell, filament, and invade a secondary host cell (Anderson et al. 2004). Re-invasion is type 1 pilus dependent both inside and outside the host cell. One study found that UPEC cannot successfully arrange in a biofilm-like community or change their morphology without the expression of type 1 pili (Wright et al. 2007). UPEC re-invasion also depends on the chemical and physical environment of the host bladder. In the presence of highly concentrated urine, UPEC was found to display close to 100% filamentation. One study concluded that surface attached filamentous UPEC are able to resist strong liquid sheer forces. Thus, the filamentous morphology gives bacteria an advantage through increased adhesion capacity during re-entry into a secondary host cell (Young 2006; Andersen et al. 2012).

In response to IBC formation, the mammalian host immune system exfoliates the superficial lining of the bladder. This now exposes the underlying, undifferentiated cells to UPEC colonization. Once internalized within the underlying cells, they are able to establish quiescent intracellular reservoirs (QIR) exclusively within endosomal vesicles. These vesicles contain about 4-10 non-replicating bacterial cells that can remain viable for months (Flores-Mireles et al. 2015). In mice, it was found that partial bladder exfoliation leads to the differentiation of underlying cell layers. As the underlying transitional cells containing QIRs differentiate, the bacterial reservoirs become

effectively activated, leading to the establishment of IBCs, and the cycle continues. (Mysorekar and Hultgren 2006).

Figure 2: The initiation of acute UPEC infection within umbrella uroepithelial cells and formation of quiescent reservoirs within underlying cells (Mulvey et al. 2000).

UPEC Proliferation and the Actin Cytoskeleton

The intracellular location of UPEC has an effect on its survival and proliferation. Iron is a limiting resource and must be acquired from within the host cell for successful pathogenesis (Skaar 2010). One study found that host cells containing IBCs increased the expression of transferrin receptors (TfR). Transferrin is a protein that is important in transporting iron ($Fe^{3+}$) in the bloodstream. Nearly every cell type expresses the TfR receptor in order to acquire iron (Zhao and Enns 2012). Intracellular TfR trafficking is mediated by Rab GTPases. Various other intracellular pathogens selectively recruit Rab proteins in order to acquire iron (Mizuno-Yamasaki et al. 2012). Some pathogens recruit Rab to prevent lysosomal degradation of their own intracellular niches (Brumell and Scidmore 2007).

Recent studies have identified Rab35, a protein that is involved in the endocytic recycling pathway. Rab35 is important to recruitment of TfR receptors to the cellular surface. Once transferrin binds to the TfR complex, Fe3+ is released into the early endosomes. Rab35 aids with the recycling of the transferrin complex to the cell surface to continue iron uptake. In mouse models, it was noted that UPEC also recruits Rab35 to

support intracellular survival. Rab35 involvement leads to an increase of TfR receptors, and thus, an increase in iron acquisition. Rab35 also was found to prevent the fusion of UPEC containing vesicles with degradative lysosomes. Collectively, both Rab35 and TfR are key features in UPEC intracellular survival (Dikshit et al. 2015).

The extent and location of UPEC infection within host cells is dependent on the actin cytoskeleton. One particular study aimed to disrupt the host F-actin in the presence of UPEC-containing late endosome-like compartments *in vitro*. It was previously understood that UPEC within these endosomes were surrounded by a complex network of actin filaments and replication was restricted. Cytochalasin D, an established inhibitor of F-actin polymerization, was added in order to disrupt the cytoskeleton (Fenteany and Zhu 2003). As a result, UPEC infection was enhanced and the bacteria divided rapidly within the cytoplasm. As in Figure 3, the authors also noted that there was an increase in actin localization near the LAMP-1 vesicle. Interestingly, other intracellular pathogens, such as *Salmonella typhimurium*, require a functional host cytoskeleton to be able to infect (Meresse et al. 2001). This is not the case for UPEC, as it seems that the actin cytoskeleton may act as a physical barrier and impede on intracellular infection (Eto et al. 2006).

Figure 3: A representation of UPEC location and efflux mechanisms during the infection process. Note that F-actin is more concentrated at the QIR sites than IBC bacterial factory sites (Eto et al. 2006).

<u>Mechanical Signaling and Integrins:</u>

Integrin and other surface receptors relay information to the actin cytoskeleton, inducing conformational changes. This interaction involves ECM proteins and the transmembrane integrin family in order to modulate cell development and gene expression (Pelham and Wang 1997). Previously, there have been several interesting studies on mammalian cell attachment and the resulting gene expression. In melanoma cells, stimulation of the $\alpha_v/\beta_3$ integrin resulted in the expression of type IV collagenase, effectively increasing the invasive ability of the cells. Monocytes undergo a rapid and global change in gene expression upon attachment to an ECM coated substrata (Juliano and Haskill 1993). Although it is well known that several mammalian cell types respond similarly to adherent interactions, the acute signaling and exact transcriptional changes have yet to be understood.

The integrin family of transmembrane proteins contains a large extracellular subunit, a single region within the plasma membrane, and a small cytoplasmic domain. Collectively, they transmit information from the ECM to the cytoplasm and vice versa. The alpha and beta subunits found within the ECM domain make up the receptor in which specific ligands are able to bind. Some receptors bind to a single ligand, whereas others bind to several. Although it is common for the ECM domain to contain the same alpha/beta sequence and structure, the cytoplasmic domains may vary. This allows for the possibility of a wide array of cellular and translational responses.

The plasma membrane of typical mammalian cells contains many integrin receptor systems that bind to ECM components. The binding of ECM proteins and integrins mediate cellular adhesion and signaling transduction through interaction with the cytoskeleton and various signaling molecules. One particular signaling molecule,

focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is one of several molecules that are localized within focal adhesions. Focal adhesions assemble in discrete locations near the cytoplasmic component of integrin proteins and act as mediators between integrins and the cytoskeleton. In this way, they are the ideal checkpoint controlling inner and outer mechanical transduction. Numerous studies have noted that both focal adhesions and FAK are critical mediators of integrin mechanotransduction. One particular study subjected various mammalian cell lines to micromanipulation through use of magnetic beads. After the mechanical stretching and pulling of the cell, it was found that there was a net increase in the size of focal adhesion (Zebda et al. 2012). This study suggests that focal adhesions play a role in eliciting mechanical signals from the ECM to the cytoplasm.

Focal Adhesions and FAK:

Focal adhesions and focal complexes mediate adhesion signaling and act as cytoskeletal organizing centers. Focal adhesions are large assemblies that transmit signals between the ECM and the cytoskeleton. They maintain a diverse array of responsibilities within the cell such as cell anchoring and transmitting signals relating to cell motility, adhesion, and the cell cycle. The basis for focal adhesion structural assembly requires the binding of focal adhesion kinases to the integrin-adapter protein-cytoskeletal complex (Zaidel-Bar et al. 2004).

FAK can be subdivided into 3 distinct parts: the catalytic domain, the N-terminal, or FERM, and the C-terminal, or FAT. Two particular tyrosine residues (Y576 and Y577) are part of the FERM activation catalytic loop and are vital for the overall activation of FAK. The formation of these complexes is mediated by cell attachment to ECM proteins

through activation of integrins. Once mature, these complexes will increase FAK's catalytic loop activity by inducing autophosphorylation at the Y397 tyrosine. The conformation of FERM is now modified so that Src-family kinases can now increase phosphorylation of the FAK complex hot spots. This site-specific phorphorylation is also triggered by mechanical forces on the surface of the outer membrane, as previous studies have noted FAK activation in fluid shear stress and cyclic stretching (Zebda et al. 2012). The focal adhesion complex is then localized deeper into the cytoplasm through a Rho-GTP mechanism. The complex then serves as a signaling center that is essential for targeting actin cytoskeletal rearrangement (McKayed and Simpson 2013).

Cell Spreading

Following the binding of certain integrin receptors, focal adhesion complexes begin to assemble near the receptor site. Actin rearrangement near the leading edge of the cell mediates focal adhesion assembly, and thus impacts cell motility and spreading. Cell spreading and motility is important in various global physiological processes such as cell growth and the organization of tissues. It is both actin and myosin dependent and results in membrane extension along the ECM surface. Spreading consists of three phases: basal, fast, and contractile. The basal phase is characterized by transient extension periods, where spatially and temporally limited cell protrusions spread in randomly determined directions. The fast phase is isotropic spreading, meaning that the cell spreads uniformly in all directions. The contractile phase is dependent on integrin-cytoskeletal signaling. Previous studies have noted that an increase in the concentration of focal adhesion complexes near the leading edge of the cell increases cell spreading. Collectively, cell

spreading and motility depend on both the cell and the environment's biophysical and molecular properties (Giannone et al. 2004; Dubin-Thaler et al. 2004; Xiong et al. 2010).

Mechanical Signaling and the Cytoskeleton:

The actin cytoskeleton is especially important to integrin signal transduction due to its involvement in cell attachment and reorganization. Talin, a cytoskeletal linker protein, contains receptors for both actin and integrin binding sites. It carries its own force of 7-10 piconewtons during cellular adhesion and is able to measure extracellular substrate rigidity (Austen et al. 2015). First, it interacts with the cytoplasmic domain of an integrin receptor. This leads to the activation of mature focal adhesions by recruiting various components to the inner membrane. Cortical tension, or the cell surface tension due to the contracting of microfilaments within the cortex of the cell, is a common cellular configuration caused by the rounding of a cell. The actin cytoskeleton can either contract or relax, depending on the amount of crosslinking proteins within the cytoskeleton network. This rearrangement is mediated by the rate of actin polymerization/depolymerization. Alternatively, the cell can emit tension on the substrate to create a flatter morphology. Previous studies have noted that actin rearrangements to place tension on the surface substrate is only possible when focal adhesion complexes are mature and integrin interactions are robust (Gauthier et al. 2012). Several observations have been made that suggest that cell attachment interactions involve not only receptor-ligand binding, but also physical communication with the cytoskeleton. Cells are able to respond to certain forces exerted from fluid sheer, adhered beads, and differing substrates. A majority of eukaryotic cells attach to soft surfaces that have an elastic moduli of 10-10,000 Pa, rather than plastic or glass materials typically used *in vitro*. The

forces generated by the cytoskeleton seem to be substrate-dependent, as they are unable to manipulate attachment sites on stiff surfaces. Fibroblasts have been noted to maintain a flatter and broader morphology upon attachment to a rigid substrate (Yeung et al. 2004). As the cytoskeleton is vital in cell morphology and physiology, it is important to rectify the relationship between attachment, mechanical signaling, and the cytoskeleton.

Actin polymerization is also a driver of cell spreading and locomotion. Cell spreading is the physical flattening and distortion of the overall cell shape. After a cell binds to a surface, several anchoring proteins are recruited to the adhesion site. These proteins lead to the maturity of focal adhesions, which then communicate with the actin cytoskeleton (Cavalcanti-Adam et al. 2007). Specifically, Rac1 and Rho trigger actomyosin contraction and protrusion, which changes the overall cell shape. This polymerization and depolymerization of the actin cytoskeleton modulates focal adhesion dynamics. The focal adhesion complex is thought to act as a cytoskeletal organizing center that controls both cellular attachment and adhesion-mediated signaling transduction (Geiger and Bershadsky 2001).

The involvement of the actin cytoskeleton in bacterial internalization was confirmed through the addition of an F-actin inhibitor. The inhibition of F-actin lead to increased bacterial escape from LAMP-1 vesicles and increased cytoplasmic replication (Eto et al. 2006). It is also well known that human cells respond to substrate compliance through activation of integrin receptors, focal adhesions, and leads to changes in the actin cytoskeleton. Actin is involved in various cell processes such as endocytosis and vesicle trafficking. Upon the initiation of a urinary tract infection, bacterial endocytosis requires host cell actin rearrangement in order to become internalized. Due to this dependence on

the host cytoskeleton, it is possible that substrates of differing stiffnesses may have an effect on the infection process. Thus, urinary tract infections are an interesting candidate to explore the relationship between substrate and infection.

UPEC Location in Cell Culturing:

In summary, IBCs form in the superficial layer of the uroepithelium and QIRs form in the underlying layers once the superficial layer has been exfoliated. Within the superficial, umbrella cells, UPEC rapidly replicates within the host cytoplasm and cycles through 4 distinct stages that lead to acute cystitis (Flores-Mireles et al. 2015). The host immune system responses by shedding the umbrella lining of the bladder, leading to the exposure of underlying bladder cells. UPEC cells are able to become internalized within these underlying layers and reside within LAMP-1 vesicles. Between 4 and 10 metabolically inactive bacterial cells can remain within these vesicles for months at a time (Mysorekar and Hultgren 2006). As the underlying, immature cells begin to differentiate into superficial cells, the actin cytoskeleton is considerably diminished. The process of cell differentiation can also trigger IBC formation from activated QIRs. (Eto et al. 2006). Interestingly, cell culture models of infection have noted that IBCs form within LAMP-1 vesicles, similar to QIRs. This particular study used PC07i cells, which is an immortalized human bladder epithelial cell line derived from the pediatric human bladder (Berry et al. 2009). Mouse models of IBC formation have noted that IBCs replicate within the cytoplasm. However, *in vivo*-like IBCs have not been observed in cell culture models. It is possible that the immortality of the cultured cells may cause the difference in location of UPEC. However, it was found that the disruption of actin leads to increased cytoplasmic infection (Eto et al. 2006). Since actin rearrangement is involved in the

persistence of cystitis and substrate stiffness induces changes in the actin cytoskeleton, it is possible that substrate stiffness may also impact the infection process of UTIs.

## Materials and Methods

### Cell lines and Bacterial Strains:

UTI89, a UPEC strain isolated from an acute bladder infection, was used for bacterial inoculation. The strain contained a GFP marker that expresses GFP uniformly for microscopic identification (Wright et al. 2005). The GFP inserted construct also contains an ampicillin and kanamycin resistance marker. 5637 (ATCC® HTB-9™), a strain of immortalized human bladder epithelial cells, was grown to confluence, where 80% of the surface of the substrate was covered with cells. RPMI media with 10% FBS was used to culture the human bladder cells. Cells were incubated at 37ºC with 5% $CO_2$.

### Infection Protocol:

In 6 well plates, 750,000 5637 cells were seeded overnight to confluence. Depending on the experiment, wells contained either soft or stiff gels (80 Pa or 10,000 Pa) or 1 µg/cm$^2$ of collagen was added directly to the empty plastic well. Wells were then inoculated with UTI89 with a GFP insert at an MOI of 1:25 by adding 100 µl of 0.5 at OD600. The plates were centrifuged at 600 g for 5 minutes to initiate bacterial contact with the host cells. Plates were incubated at 37ºC for 2 hours then aspirated, washed, and replaced with media containing a high concentration of gentamicin (100 µg/ml) for 2 additional hours. 4 hours post inoculation, the media was replaced again with a low concentration of gentamicin (15 µg/ml) overnight unless specified otherwise.

### Gentamicin Protection Assay

Confluent 5637 cells were inoculated with WT UTI89 or UTI89 with the *fim* promoter switched off (Greene et al. 2015). The *fim* off strain does not express type 1 pili

and therefore, should not be able to adhere to the surface of the bladder cells and infection. 2 hours post inoculation, samples were washed 3 times with PBS then gentamicin was added to the media at a concentration of 100 µg/ml for 2 hours. At 4 hours post infection, the high concentration of gentamicin was replaced with a low concentration at 15 µg/ml and incubated overnight. 24 hours post infection, the samples are lysed in 0.2% Triton-X100, diluted, and plated on LB plates with kanamycin.

Polyacrylamide Gels

In order to maintain substrates of different compliances, polyacrylamide gels were constructed on glass coverslips (Figure 4). First, coverslips were chemically modified to react with a mixture of acrylamide, bis-acrylamide, and a succinimidyl linker as previously described (Yeung et al. 2005). Gels were then washed and collagen was added at a concentration of 1 µg/cm$^2$ to coat the gel and act as an extracellular matrix to which the bladder cells can attach. 5637 cells were then seeded on the gel coverslips and grown to confluence overnight. In 6 well plates, 750,000-1,000,000 bladder cells were seeded overnight.

Figure 4: Protocol for the construction of polyacrylamide gels of varying stiffnesses.

Figure 5: Previous determinations of gel stiffness were used to control the stiffness of each gel by varying the ratio between acrylamide and bis-acrylamide. The red circle is the concentration used to construct soft gels of 80 Pa and the black circle is the concentration used to construct to stiff gels. Each line represents the percentage of

acrylamide, while the x axis % crosslinker is the percentage of bis-acrylamide. The y axis denotes the corresponding shear modulus in Pascals. Stiff gels were prepared with 7.5% acrylamide and 0.3% bis-acrylamide, to result in 10,000 Pa. Soft gels were prepared with 3% acrylamide and 0.04% bis-acrylamide, to result in 80 Pa (Yeung et al. 2005).

Immunofluorescence

Each infected well contained either a plain glass coverslip or a polyacrylamide glass coverslip of diameter 25 mm. Plates were incubated for 2 hours, 24 hours, 48 hours, or 72 hours. The samples were then washed 3-5X before being fixed in 3.7% formaldehyde for 10 minutes. Fixed samples were quenched with 0.1 M $NH_4Cl$ for 10 minutes. Cells were then transferred to a moist chamber, where 0.2% Triton-X100 was added for 10 minutes to permeabilize the cells. Samples were then blocked with 2% BSA for 30 minutes. 500 µl of 1X PBS was added to the top of the coverslip for 10 minutes 3 separate times. A Santa Cruz Biotechnology® mouse anti-LAMP-1 specific antibody (lot # HO614) was added at a concentration of 1:200 for one hour. The coverslips were washed 3X with 1X PBS, then a Santa Cruz Biotechnology® secondary anti-mouse antibody was added at a concentration of 1:1000. Once cells were washed again, Alexa648 conjugated phalloidin and DAPI were added in the dark at a concentration of 0.5 µm and 300 nM, respectively, for 10 minutes. Finally, cells were briefly washed and placed inverted on an additional glass coverslip using SlowFade® Diamond anti-fade mountant. Immunofluorescent images were photographed and analyzed using a Leica confocal microscope (Leica Microsystems Inc. Buffalo Grove, IL, USA).

Infection Quantification

To quantify invasion of UTI89 within human bladder cells, gentamicin-treated infected cells were washed briefly and lysed in 500 µl of 0.1% Triton-X100. After 10 minutes, 300 µl of each sample was removed to be used for bacterial quantification. They were quantified by serial dilution and plating on LB plates with kanamycin overnight. The remaining 200 µl was used for genomic DNA isolation through use of the Zymo Quick-gDNA™ MicroPrep kit, where the cell suspension sample protocol was used. The genomic DNA was then quantified using QPCR which targeted GAPDH to identify the presence of bladder cells. To generate a standard curve for quantification, uninfected 5637 cells were counted, genomic DNA was isolated, and several DNA dilutions were analyzed by QPCR. The standard curve was used to calculate the number of host cells within each sample. The CFU/ml was calculated and combined with the amount of host cells. The final result was an infection ratio that expresses the amount of bacterial cells per host cell. The gel samples were compared with plastic samples that received the same treatments.

**Results:**

Confirmation of the internalization of UPEC within host human bladder cell:

In order to examine the infection process, internalization of UPEC within host cells needed to be confirmed. Internalization of UPEC is mediated by host integrin-cytoskeletal complexes, which may have been effected by the immortalization of the 5637 cell line. Using confocal microscopy, a 3D image of the infection process was analyzed. Figure 6 represents the infection on glass coverslips. It is difficult to discern whether UPEC is within the host cells, or on the surface, as the cell membrane was not able to be stained.

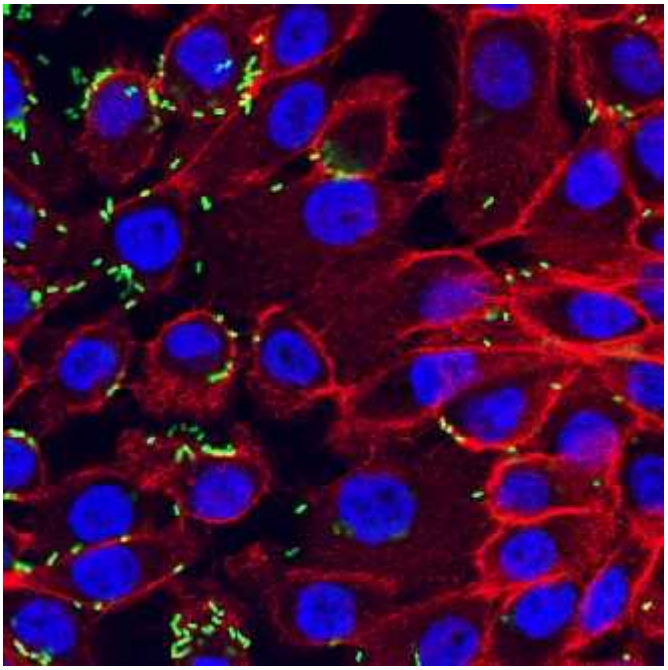


Figure 6: Immunofluorescence of 5637 UTI89 infection on glass coverslip at 24hrs. 5637, nuclei were stained blue with DAPI; 5637 cytoskeleton was stained red with phalloidin; and UTI89 which expresses GFP that allowed for the bacteria to be imaged. Several 3D images were taken to confirm the internalization of UTI89 as well as to make sure the infection process was occurring as previously described.

To further verify bacterial internalization, the gentamicin protection assay was performed. Gentamicin is a bactericidal antibiotic that is unable to penetrate the cell membrane of mammalian cells. The host cells were infected with either wild type UTI89 or UTI89 with the *fim* promoter flipped in its inactive orientation. The *fim* gene mediates the expression of type 1 pili, the primary adhesion molecule of UPEC. Intracellular bacterial organization and aggregation have been found to be type 1 pili dependent as well. Therefore, the *fim* off sample does not have the capability to invade the host cells, and should be killed in the extracellular space due to the presence of gentamicin. Once the infection period was complete, host cells were lysed to release all internalized bacteria. As seen in Figure 7, results revealed that the wild type samples contained a significant amount of colonies when compared with the very few colonies in the mutant

samples. The gentamicin protection assay confirmed the effectiveness of gentamicin and the internalization of UPEC within the uroepithelial host cells.

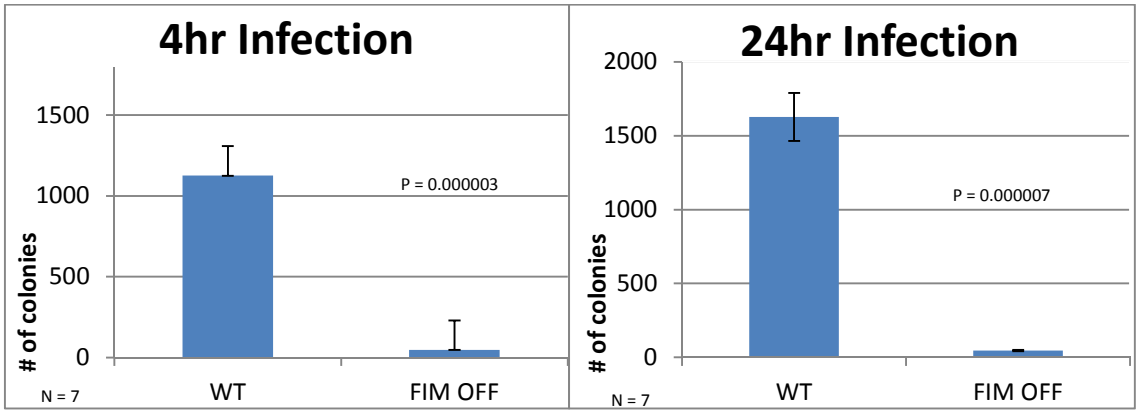
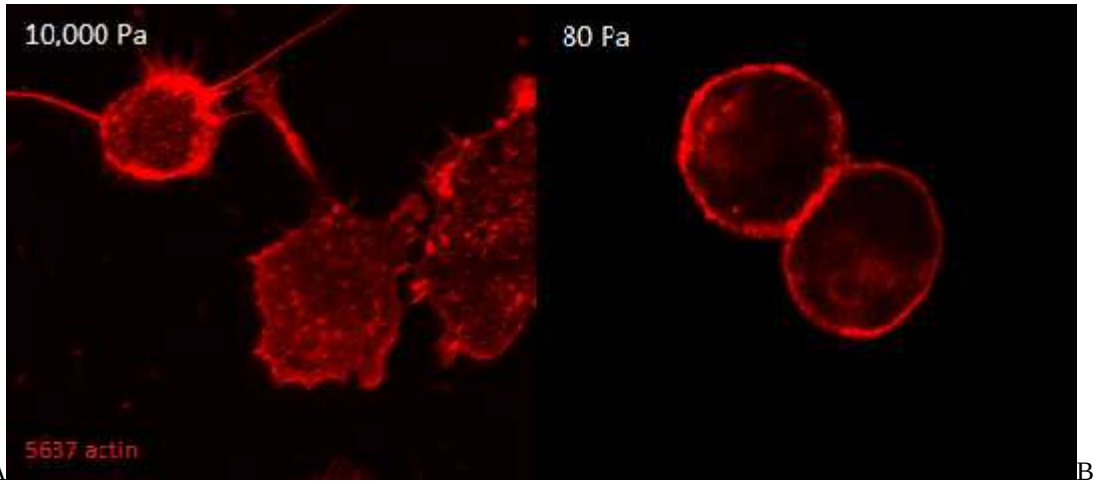



Figure 7: A gentamicin protection assay was performed to quantitatively confirm the internalization of UTI89 within human bladder epithelial cells. After 4 and 24 hours post inoculation, the wells were lysed and plated on LB plates. The WT sample contained significantly more colonies than the *fim* off strain. This confirms that the WT bacteria are able to enter the host cells, effectively being protected from the gentamicin antibiotic.

Morphological changes of uroepithelial cells on polyacrylamide gels of varying compliance:

To further explore the effect of substrate stiffness on uroepithelial cells, host cells were seeded on coverslips of varying stiffnesses. As represented in Figure 8, the host cytoskeleton differs between three gels of ascending compliance. In Figure 8A, the host cell cytoskeleton is well spread. Eukaryotic cells spread to acquire a flattened morphology, which is mediated by integrin signaling and intracellular cascades involving tyrosine kinases and protein kinases (Price et al. 1998). The binding of integrin α3β1 receptors to the ECM typically induces the assembly of focal complexes and focal

adhesions. Small GTPases Rac1 and Rho trigger actin polymerization and actomyosin contractility, leading to dramatic changes in cell shape and spreading (Cavalcanti-Adam et al. 2007). In Figure 8B, cells do not appear to be well spread and lack actin stress fibers. This is consistent with previous studies that found that actin stress fibers were present only while attached to stiff surfaces (Yeung et al. 2005). There is a clear difference in the cytoskeletal arrangement within host cells that are attached to various compliances.

A 

B

Figure 8A and B: Human uroepithelial cells were seeded on 2 different gels and were imaged using confocal microscopy. All samples were stained with phalloidin to observe the actin cytoskeleton. The cells in image A were seeded on the stiffest gel and appeared to have a more flattened and spread morphology than image B. Image B was seeded on the softest gel and appeared to have a rounder morphology compared to the stiffest gels. There seemed to be little to no spreading and stress fibers were absent. The aim of these images was to confirm that there is a difference in uroepithelial morphology upon attachment to polyacrylamide gels of descending stiffnesses.

<u>Arrangement of uroepithelial cells differs on soft gels:</u>

Uroepithelial cell arrangement and aggregation also differs significantly on soft gels when compared with stiff gels or glass/plastic. The images in Figures 9 and 10 represent a spherical collection of various host cells on a soft substrate. Unlike typical monolayer cultures, the cells on soft gels arrange in a 3D structure. This 3D arrangement was not found in gels of medium stiffness, high stiffness, or glass/plastic. In mammary epithelial cells, an initial spherical shape is a critical step in development and maintenance of polarized differentiation. Loss of polarity has been linked with increased cell proliferation and tumorigenesis (Debnath et al. 2003). Recent studies have noted the importance in cell-cell adhesion in the formation and maintenance of a 3D spherical arrangement, and thus, cell polarity (Bissell, M. J. and Radisky, D. 2001).

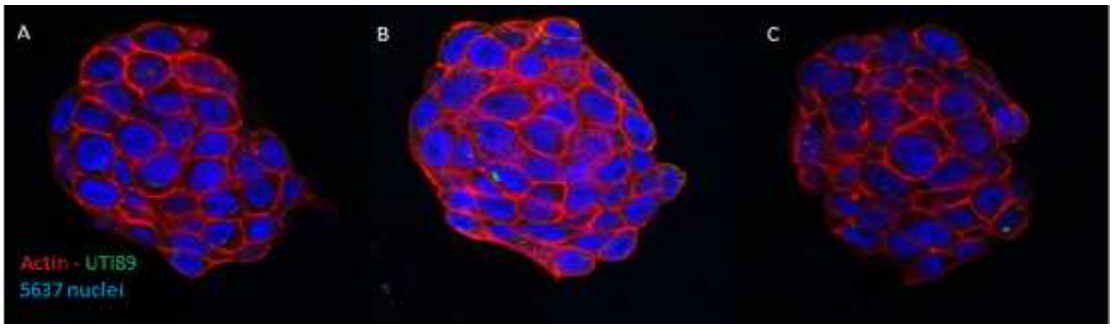



Figure 9 A, B, and C: Uroepithelial cells infected with UTI89 on soft polyacrylamide gels of 80 Pa. These images were part of a single Z stack and are sequential from bottom to top of the cell aggregate. There is no spreading within these cells and they are arranged in a large spherical mass. Previous studies have noted that mammary epithelial cells arrange in a similar 3D sphere *in vivo* (Debnath et al. 2003).

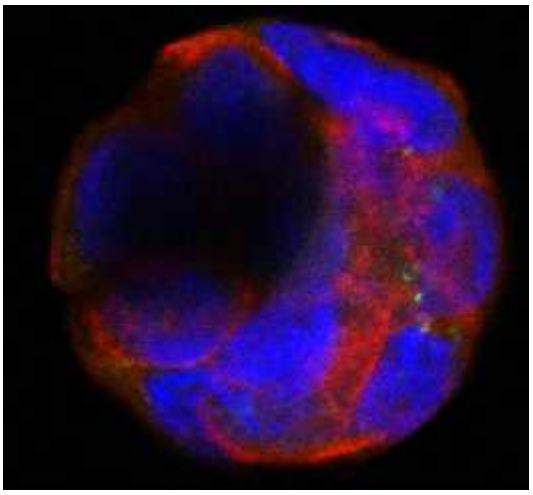


Figure 10: 3D mass of infected uroepithelial cells on soft gels of 80 Pa. (N = 12). The Z stack in which this image was taken shows that the cells are arranged in a thick, 3D sphere. There were no similar cellular arrangements found on stiff gels or plastic substrates.

Location of UPEC during uroepithelial invasion:

*In vivo* UTIs within the superficial umbrella layer of the uroepithelium form complex intracellular bacterial communities (IBC). Upon internalization, UPEC is immediately found within Rab27b/CD63 secretory vesicles that aim to expel the bacteria to prevent infection. However, some bacteria are able to escape the vesicle before exocytosis occurs. The escaped bacteria are able to replicate within the cytoplasm and rapidly organize into IBCs. In response to umbrella cell invasion, the host exfoliates the layer of superficial umbrella cells. The loss of this differentiated, superficial layer leads to the development of QIRs within the underlying, undifferentiated cells. QIRs are small collections of bacteria that lie metabolically inactive within LAMP-1 vesicles. Previous studies have noted that *in vitro* infections result in IBC-like aggregates that differ from *in vivo* infections in the location of UPEC. Instead of proliferating within the host cytoplasm, the bacterial factories are found in LAMP-1 vesicles (Berry et al. 2009). As seen in Figure 14 C and D, bacterial factories were found within LAMP-1 vesicles on stiff gels. A similar phenomenon was found on plastic Petri dishes (Figure 14 A and B). In contrast, the bacteria were found to be located within the cytoplasm on the softest gels

of 80 Pa in Figure 14 E and F. Although the mechanism by which UPEC escapes vesicles is unknown, it is assumed that following internalization/endocytosis on soft gels, some percentage of bacteria must be able to escape and proliferate in the cytosol. The dominate pathway that induces bacterial exocytosis out of the host cell and into the extracellular space involves cyclic AMP and calcium levels (Bishop et al. 2007). Previous studies that focused on FimH mutants found a correlation between cAMP levels and bacterial vesicle escape. Therefore, it would be interesting to examine cAMP and calcium levels in soft gel UPEC infection in future studies (Wright et al. 2007).

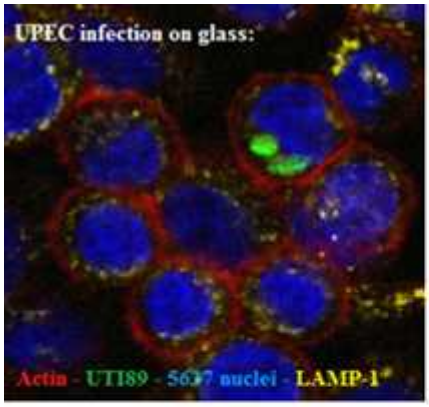
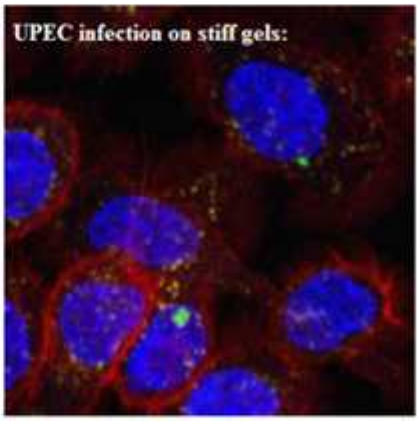



Figure11:Immunofluorescent staining of UTI89 infection within 5637 human uroepithelial cells on glass coverslips. Overlay of infection; uroepithelial cell were stained yellow using a LAMP-1 specific antibody. The bacterial factory is localized within a LAMP-1 positive vesicle.

Figure12: Immunofluorescent staining of UTI89 infection within 5637 human uroepithelial cells on stiff polyacrylamide gel coverslip of 10,000 Pa. Cells are well spread and have a flattened morphology.



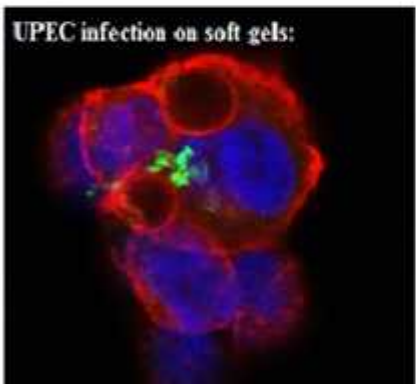




Figure 13: Immunofluorescent staining of UTI89 infection within 5637 human uroepithelial cells on soft polyacrylamide gel coverslip of 80 Pa. Overlay of infection; uroepithelial cells are round and do not spread.

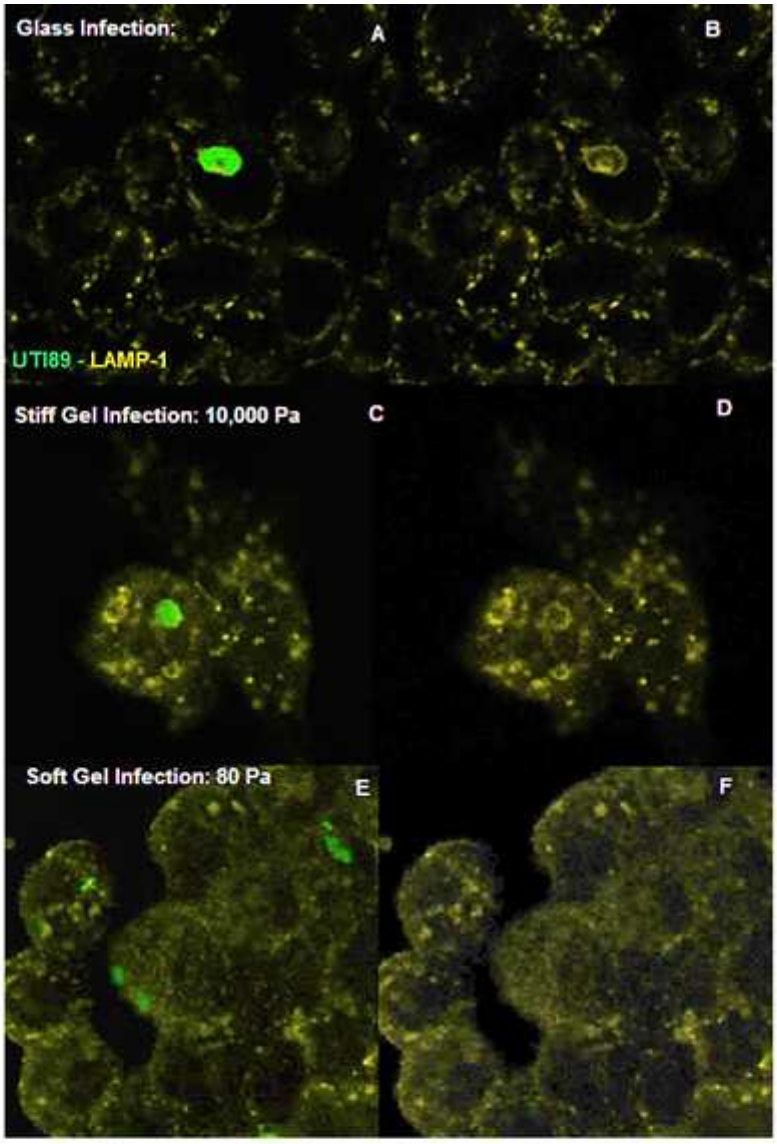



Figure 14: A-F: Comparison of the location of UTI89 bacterial factories within the uroepithelial cell on glass, stiff gels, and soft gels (N=41, N=49, N=38). In the glass and stiff gel infections (A-D), the bacterial factories were found to be associated with LAMP-1 positive vesicles. Image A represents glass infection with the bacterial factory and LAMP-1 staining only. Image B represents the same image as A, except without the bacteria. In image B, there is a ring of LAMP-1 surrounding the area where the bacteria were originally imaged in A. Image C represents the infection on stiff gels and displays the LAMP-1 staining and bacterial factories. Image D is the same as image C, but it displays the LAMP-1 staining only. There is a distinct ring of LAMP-1 around the area in which the bacterial factory occupies. By comparison of image A/B and C/D, the bacterial factories are associated with LAMP-1 vesicles. Similar comparison of images E and F shows that the bacteria is not associated with LAMP-1 vesicles.

UPEC filaments on soft gels:

Previous studies have noted that UPEC morphological plasticity may also be an intra and extracellular survival strategy. A defining characteristic of IBC formation is the ability of UPEC to change its overall cell shape. Studies suggest that the induction of morphological differentiation is a type 1 pili-dependent response to physical and chemical cues in the bladder. As seen in Figure 15, UPEC filaments in the late stages of IBC formation. As seen in the image, at the filamentous step, UPEC escapes from its original host cell and aims to become internalized within a secondary cell. It was found that UPEC increases its adherence ability while filamentous, suggesting that this morphological change may be a response to the extracellular bulk flow of urine. To our knowledge, there has yet to be any cell culturing studies that have noted the presence of filaments and secondary cell infection (Czaja et al. 2009; Andersen et al. 2012). This may be due to the organization of the actin cytoskeleton. As seen in Figure 8A and B, focal adhesions and actin stress fibers are not present on soft gels. On stiff gels and glass/plastic substrates, cells are well spread and have a flattened morphology. These changes in overall cell shape are mediated by integrin/focal adhesion complexes. UPEC attachment to the host cell surface requires interaction between UPEC FimH adhesin and manosylated uroplakin/α3β1 integrin receptors (Sun et al. 2010). It is possible that this interaction is disrupted on stiff and inflexible substrates, leading to the inability of UPEC to complete the filamentous step in IBC formation.

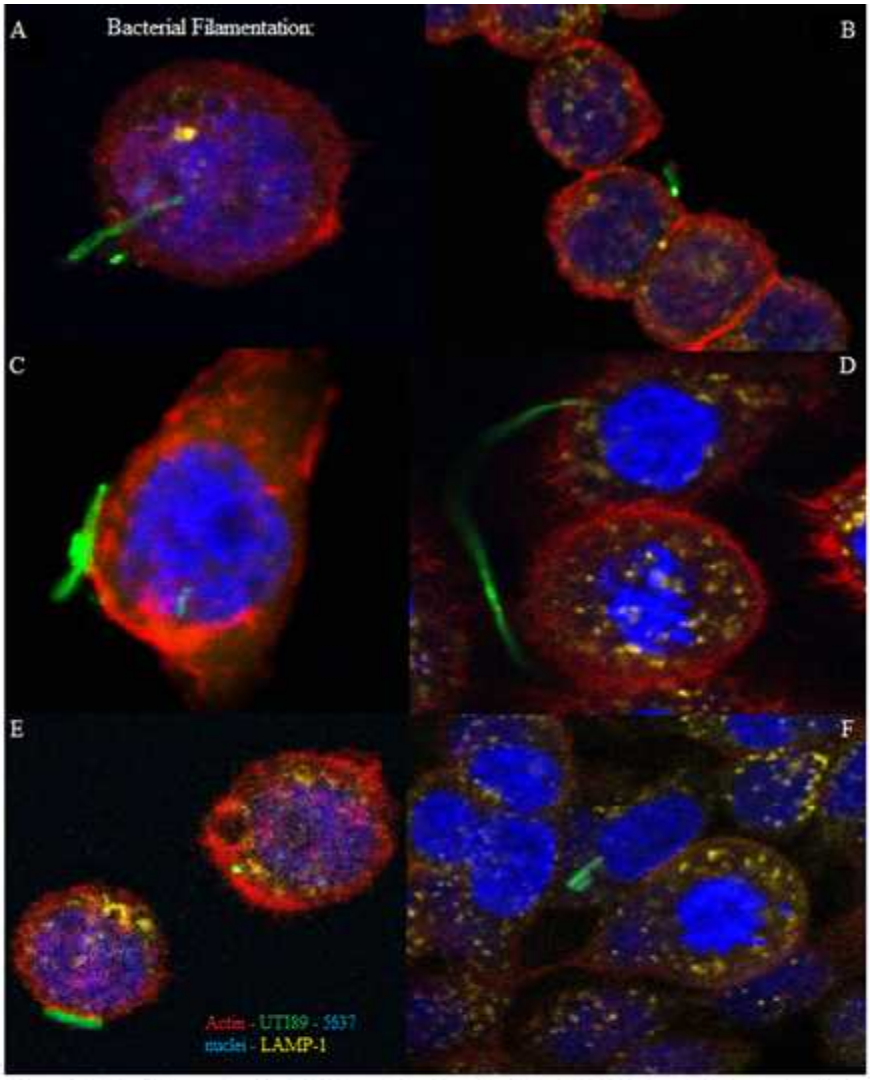



Figure 15: Uroepithelial cell infected with UTI89 on soft polyacrylamide gel of 80 Pa at 24 hours after invasion (N=7). Filamentation of UPEC was found regularly on soft gels, but was not seen on stiff or glass coverslips. During IBC formation, UPEC becomes filamentous in order to make contact with surrounding host cells and persist the infection process.

Quantification of uroepithelial UPEC infection on polyacrylamide gels:

Although the confocal images have revealed interesting differences in UPEC infections on various substrates, infection quantification from images alone posed to be difficult. When the same numbers of host cells are seeded on each of the gels, a different amount of cells are able to adhere to the ECM surface of the gels. Bacterial factories are a collection of replicating bacteria and are difficult to quantify from images alone. Instead,

a combination of QPCR and bacterial titers were used to explore the amount of UPEC cells per host cell. DNA was first extracted from a known amount of host cells and diluted. Each dilution represents a certain amount of counted host cells. The standard curve in Figure 16 was used to calculate the number of host cells from the corresponding CT in each of the unknown samples. Half of each sample was also lysed and plated on LB plates to calculate colony forming units. These results were then combined to quantify the amount of bacteria per host cell, or an infection ratio. The first step was to confirm this method by examining UPEC infection samples on plastic substrates. Once the infection ratio was confirmed, stiff and soft gel samples were calculated to identify a variance between the two substrates. As shown in Figure 17, the softest substrate contained the most bacteria per host cell.

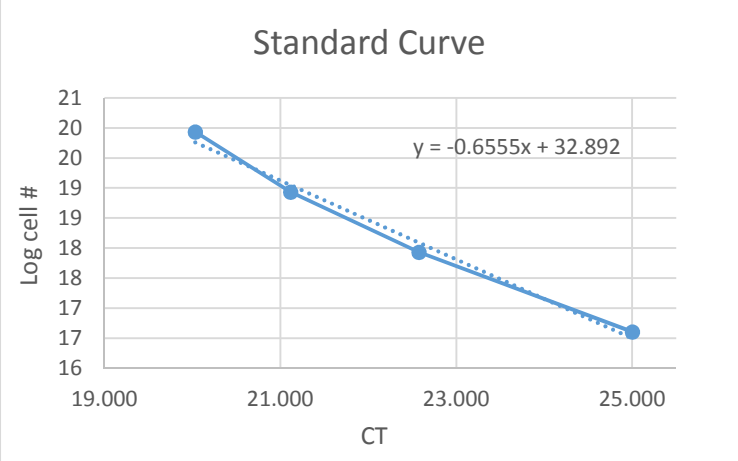



Figure 16: Standard curve of CT values versus the logarithm of known amounts of uroepithelial cells. Host cells attach to the gels and plastic at different rates, thus the standard curve allows for relative quantification. In order to measure the number of attached cells, QPCR is used to target GAPDH, a mammalian gene that is indicative of the presence of uroepithelial cells. A standard curve is then constructed in order to

correlate CT, or the relative amount of DNA, with a known amount of suspended uroepithelial cells.

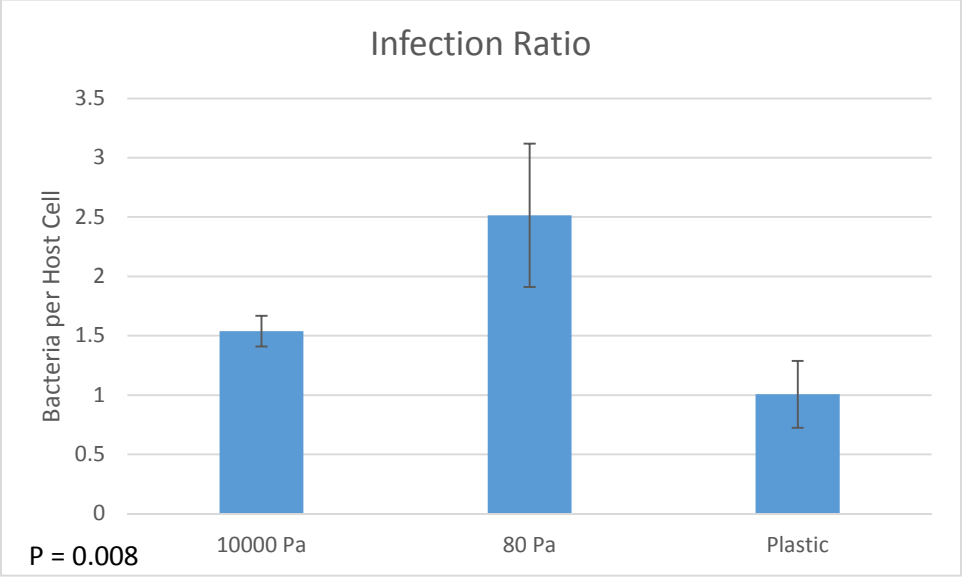



Figure 17: Quantification of UPEC infection of uroepthelial cells on stiff and soft gels and a plastic dish (N=10). The stiff gel is 10,000 Pa, whereas the soft gel is 80 Pa. The infection ratio, or number of bacterial cells per host cell, was obtained using a standard curve as described in figure 16. The data suggests that there are more bacterial cells per host cell on soft gels when compared with a stiff gel or a plastic substrate.

**Discussion:**

Previously, it has been established that the substrate in which a eukaryotic cell is attached has an effect on cell morphology and physiology. Various integrin surface receptors receive signals from the substrate in the ECM and relay the signal to the interworking of the cytoplasmic tail. The process involves several pathways that eventually lead to a complex, dynamic change in gene expression and actin rearrangement. Morphological changes in mammary epithelial cells have shown to be

critical to proper development of differentiated cells. This may also be the case in uroepithelial cell arrangement in cell culturing, as differentiation plays a critical role in IBC/QIR formation.

One cell culturing study noted that the disruption of the host actin cytoskeleton leads to increased UPEC proliferation within vesicles. By contrast, other pathogens seem to require actin arrangement in order to infect, while UPEC is inhibited by the tight regulation of host actin. From Figures 8, 9, and 10 it is clear that host cells on softer substrates impact host arrangement and morphology. Vesicle trafficking is also mediated by the cytoskeleton and protein complexes. Internalization and proliferation of UPEC involves vesicle trafficking and resource acquisition. Therefore, it is possible that substrate stiffness may affect the infection process by interacting with the actin cytoskeleton.

Host cell focal adhesion complexes are able to respond to changes in the ECM, including substrate compliance. It is possible that the difference in location of UPEC within high compliance and low compliance gels may be due to changes within the actin cytoskeleton (Bishop et al. 2007; Sun et al. 2010). Interestingly, LAMP-1 vesicles are unusually stable due to extensive glycosylation and intrachain disulfide bonds. The unique stability allows them to be resistant to proteolytic degradation. A majority of the vesicles are found associated with the plasma membrane (Acevedo-Schermerhorn et al. 1997). Collectively, these properties may serve as protection for QIRs and bacterial factories *in vitro*. The environment of the intracellular bladder poses significant threats for UPEC between enzyme degradation, antibiotics, and resource depletion. UPEC may

remain in LAMP-1 vesicles in order to avoid host threats and increase survival (Bower et al. 2005).

Quantification of UPEC infection shows that there may be a difference in the infection process on substrates of differing stiffnesses. We speculate that the difference may arise from an increase in bacterial endocytosis or decrease in exocytosis within the host cells. In order to form IBCs *in vivo*, UPEC must escape secretory vesicles into the cytoplasm. The presence of UPEC in LAMP-1 vesicles in QIRs has been noted to protect the bacteria from host cell defenses and antibiotics. Both processes are mediated by the actin cytoskeleton. It may be possible that the global signaling changes induced by a soft substrate have an impact on vesicle transport and bacterial escape.

Previous studies found that actin localizes around vesicles containing bacteria, presumably to reduce bacterial growth and replication. QIRs *in vivo* are also located in LAMP-1 vesicles and are metabolically inactive. In the infections on glass, plastic, and stiff gels, bacterial factories were found within LAMP-1 positive vesicles. The gentamicin protection assay results revealed an interesting observation regarding UPEC intracellular replication. The number of colonies was essentially the same at 4 hours and 24 hours post infection. This suggests that bacterial growth was hindered in some way, considering that UPEC typically doubles its population size every 30-60 minutes in mouse bladder infections (Scott et al. 2015). In future experiments, we aim to repeat the gentamicin protection assay on polyacrylamide gels to compare an intracellular UPEC growth curve on different substrate compliances.

Future directions may focus on the impact of substrate stiffness on bacterial vesicle trafficking to clarify differences in the infection ratio. It would be interesting to

understand this process in cell culturing, as IBC formation occurs within LAMP-1 vesicles instead of the cytoplasm on stiff surfaces. Actin disturbance would also be interesting to explore on differing substrates to dissect the relationship between infection, actin, and substrate.

Cytoskeletal rearrangement and vesicle trafficking require a vast complex of proteins. In particular, the Rab27b/CD63+ vesicle that is primarily responsible for UPEC internalization is mediated by cyclic AMP. It would be interesting to clarify the role of cAMP and other small GTPases within UPEC internalization on differing substrates. Just as UPEC is able to recruit Rab35 to acquire iron for survival, it possible that the bacteria is able to effect the Rab27b/CD63+ and LAMP-1 differently, depending on the substrate compliance.

The difference in UPEC infection on differing substrates may also be due to an increase in host apoptosis. Once a host cell undergoes cell death, the internal contents are then released into the extracellular matrix. On softer substrates, it is possible that the impact on the host cytoskeleton may lead to higher host cell death through increased infection. Previous studies on cell culture IBC formation noted that UPEC is able to re-infect just as is done *in vivo*. A softer substrate may create an intracellular and extracellular environment in which the bacteria is more successful at invading due to the increase in host apoptosis. Further studies are required to attain a complete understanding of the *in vitro* UPEC infection process on varying substrates. It would be interesting to compare the amount of initially attached host cells with infected host cells to further understand the role of apoptosis and increased infection.

**Conclusion:**

The proliferation and survival of UPEC within uroepithelial cells is a complex process mediated by host actin cytoskeletal rearrangement. Uroepithelial cells are able to respond to extracellular physical cues such as substrate stiffness. UPEC infection differs on soft gels when compared with stiffer surfaces. UPEC resides in the cytoplasm, rather than the LAMP-1 vesicles, on soft gel infections. Previously, *in vivo*-like IBCs were not able to form in cell culturing studies. Our data suggests that a softer substrate is necessary to construct a normal UPEC intracellular community. The normalizing of cell cultured UPEC IBCs may be due to the effect of substrate stiffness on vesicle trafficking and bacterial escape. A quantifiable difference in infection was also noted upon soft gels. It is possible that the substrate's effect on the host cytoskeleton alters endocytosis and exocytosis enough to result in a more successful infection overall. Further cytoskeletal and vesicle trafficking studies are required to completely discern the effect of substrate stiffness on UPEC infection.

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