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Characterization of a UPEC *degS* mutant *in vitro* and *in vivo*

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Summary

***Escherichia coli* DegS is an integral inner membrane protein that breaks down incorrectly assembled proteins and helps regulate the σ^E stress response pathway. When *degS* is deleted, approximately 200-fold more outer membrane vesicles (OMVs) are produced. As OMVs contain sections of the outer membrane and proteins from the periplasm, they may play a role in the virulence of *E. coli*. To test whether *degS* or OMVs play a role in uropathogenic *E. coli* (UPEC) virulence, we characterized a *degS* mutant. We found that the *degS* deletion did not affect the morphology of the bacterium, but did result in decreased virulence *in vivo* and *in vitro*. When we tested our mutant in a murine model of cystitis, mice infected with *degS::cat* bacteria had a 100-fold decrease in bacterial titers in the bladder at 24 hours post-infection. *In vitro* binding and invasion assays showed no difference in the ability to bind to host cells, but a significant decrease in the ability of mutant bacteria to invade cells. Together, these data suggest that *degS* deletion decreases the virulence of *E. coli* and that an increase in OMV production may be a detriment to virulence. Future studies can separate the role of *degS* and OMV production in UPEC virulence.**

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Introduction

Escherichia coli is a common species of bacteria that inhabits the intestines of humans and animals. It is a rod-shaped, gram-negative, and facultative anaerobic bacterium. While most strains are harmless, some are capable of causing disease in humans. One such strain is uropathogenic *E. coli* (UPEC), the causative agent of over 85% of urinary tract infections (UTIs) (1). UTIs can affect the bladder, termed cystitis, or spread to cause a kidney infection, termed pyelonephritis. Some infections will affect both the bladder and the kidneys. Over 50% of women will develop at least one UTI in their lifetime, and many of these women will have recurrent infections that affect their quality of life (1). While there are likely many host factors that contribute to the occurrence of UTI, UPEC are characterized by the expression of specific virulence factors such as type 1 pili, flagella, hemolysin, and siderophores (2, 3). These virulence factors aid the bacteria in binding the host bladder epithelial cells. Bound bacteria can be internalized, where they are protected from antibiotics and the immune system (3). Virulence factors help the bacteria acquire necessary nutrients and grow to high titers within the cells. Some of these intracellular bacteria will remain hidden for up to 6 months before emerging from the cells and causing a recurrent infection (3). In addition to the intracellular bacteria, bacteria are also able to persist in the lumen of the bladder and can ascend up the ureters to cause pyelonephritis. The discovery of additional UPEC virulence factors could lead to strategies for the prevention of UTIs in humans and the development of more effective drugs for the treatment of *E. coli* infections.

degS of *E. coli* encodes a 355-residue protein that is a homolog of the DegP protease (4). The DegS protein is an integral inner membrane protein with an active site in the periplasm (5). This protein is able to detect and break down misfolded proteins in the periplasm so that they can be cleared from the cell (5). Additionally, DegS provides proteolytic cleavage of the RseA anti-sigma factor when misfolded proteins are detected to regulate sigma E (σ^E) activity in *E. coli* cells (5). Activity of the σ^E pathway is essential under conditions of envelope stress, as well as in non-stress conditions, because it maintains homeostasis within cells (6). When *degS* is mutated in bacteria, the production of outer membrane vesicles (OMVs) is increased by approximately 200-fold, most likely in response to the accumulation of misfolded proteins (7). While much of this original work was done in the non-pathogenic *E. coli* strain DH5α (7), follow-up

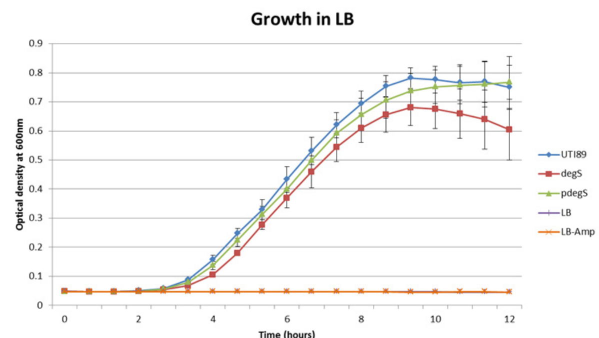

Figure 1: Growth of UTI89, *degS::cat* and p*degS* in LB broth. Optical density at 600 nm was measured every 40 minutes during growth at 37°C. UTI89 is shown in blue, *degS::cat* is shown in red and p*degS* is shown in green. LB broth and LB broth with ampicillin (used as blanks) are shown in purple and orange, respectively. The average optical density was graphed, error bars represent standard deviation.

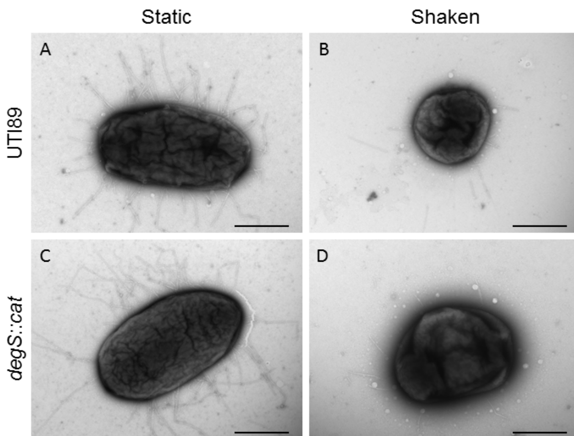

Figure 2: TEM images of UTI89 and *degS::cat*. Representative images of UTI89 and *degS::cat* cells are shown under shaken and static growth conditions. The scale bar represents 500 nm.

work confirmed that deletion of *degS* from a UPEC strain similarly increases the production of OMVs (Danka and D.A. Hunstad, unpublished).

OMVs are nano-sized (~10-300 nm in diameter), spherical, bilayered proteolipids that are produced when small portions of the bacterial outer membrane bulge away from the cell, break off, and release (8). The production of OMVs has been described as a bacterial stress response, but experimental evidence suggests that OMVs play a role in pathogenesis and are associated with virulence factors and toxins (9, 10). OMVs bear components that are thought to impact the course of infection and are likely to play a significant role in the virulence of gram-negative bacterial pathogens (9, 10). Although much work has been done on the role of OMVs in various settings, it is unknown whether OMVs are liberated by membrane instability or through a more directed process.

To investigate the role that *degS* and OMVs have on the virulence of UPEC, we characterized a *degS::cat* UPEC strain. We aimed to determine if *degS* deletion affects the virulence of UPEC. We hypothesized that *degS::cat* would be more virulent due to the increased production of OMVs, which can play an important role during infection. If *degS::cat* was more virulent, it would suggest that OMVs do increase virulence, but that *degS* is not essential for UPEC virulence. Conversely, if the wild-type strain was more virulent, this could mean that an increase in OMV production is detrimental to virulence, or that *degS* is essential for virulence in UPEC. We used a variety of *in vitro* and *in vivo* assays to compare our strains and to test the virulence of our mutant. Our results indicated that the deletion of *degS* slightly impairs growth of the bacteria, but does not change the morphology or protein expression in the membranes. Furthermore, *degS::cat* was less virulent than wild-type UPEC both *in vitro* and *in vivo*, demonstrating that the deletion of *degS*, accompanied by an increase in OMVs, decreases the virulence of UPEC.

Results

Wild-type, *degS::cat*, and p*degS* strains do not have significantly different growth in LB

Growth curves of UTI89, UTI89 *degS::cat*, and UTI89 p*degS* were developed to compare the effects that *degS* mutation has on bacterial growth. Cells were cultured in LB, and the optical density was measured every 40 minutes for 12 hours to create growth curves (Figure 1). Strains had similar growth at all time points. The mutant was less dense than the wild-type or complemented strains at most time points, although this was only significantly different at two points in early log phase.

degS::cat does not have altered morphology under aerated or nonaerated conditions

Transmission electron microscopy was used to examine the effects that shaking and static growth conditions have on the size, shape, and pili of bacterial cells from the wild-type strain or *degS::cat*. Shaken cultures of either strain produced bacterial cells that were coccoid with few pili (Figure 2B, D). Conversely, cells from static cultures were rod-shaped with lots of pili (Figure 2A, C). The wild-type and mutant strains had similar morphological characteristics in both the static and shaken cultures, indicating that *degS::cat* does not alter morphology under either condition.

Protein composition of membranes and cytosol is similar between strains

SDS-PAGE gels were used to compare the protein content of whole cell lysates of wild-type and mutant cells to see if *degS* mutation affects the expression of other proteins. There were no observable differences in the protein composition of whole cell lysates from wild-type or mutant cells when stained with Coomassie blue (Figure 3A). Cellular fractionation and SDS-PAGE gels were used to more closely examine the composition of the inner membrane, outer membrane, and cytosol of the two strains. We did not see any differences in the proteins in the outer membranes of our two strains (Figure 3B). Surprisingly, we found that *degS::cat*

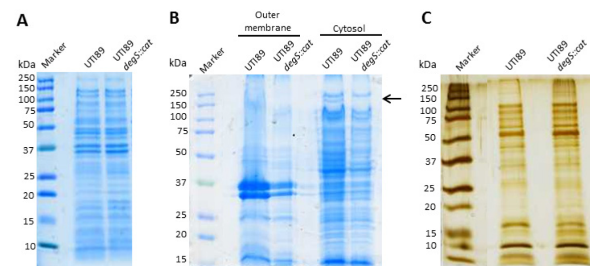

Figure 3:Protein expression of whole cells and cellular fractions. (A) Coomassie stain was used to show the protein expression in whole cell lysates from both strains. (B) Coomassie stain was used to show protein content in the outer membrane and cytosol of both strains. Arrow indicates a missing high molecular weight protein in the cytosol of *degS::cat*. (C) Silver stain was used to show the protein content of the inner membrane of the two strains. Marker indicates the approximate protein size in kDa.

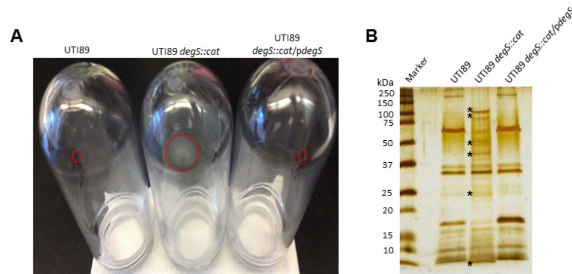

Figure 4: Protein expression in isolated OMVs. (A) Pellets of OMVs after ultra-centrifugation of filter-sterilized supernatants from overnight cultures. Pellets are circled in red. (B) A silver-stained protein gel revealed differences in the protein composition of OMVs. Examples are denoted with asterisks (*). Marker indicates the estimated protein size in kDa.

was missing a high molecular weight protein band, approximately 200 kDa, which appeared in the cytosol from wild-type bacteria; however, we were not able to identify this protein (arrow, Figure 3B). Finally, a silver-stained SDS-PAGE gel was used to analyze the protein expression in the inner membrane (Figure 3C). We did not note any major differences in the protein content of the inner membrane between the two strains.

degS::cat results in increased production of outer membrane vesicles

In order to confirm that *degS::cat* produces more outer membrane vesicles (OMVs) than the wild-type strain, OMVs were isolated from overnight cultures of the wild-type, *degS::cat*, and p*degS* strains. Visual inspection of the pellets of the OMVs recovered after ultracentrifugation revealed a much larger pellet in *degS::cat*, compared to either the wild-type or complemented strain (Figure 4A). This was confirmed by resuspending the pellets in equal volumes and determining the protein content in each (UTI89 = 31 μg/mL, UTI89 *degS::cat* = 910 μg/mL, p*degS* = 51 μg/mL). Transmission electron microscopy confirmed that these pellets contained mostly intact OMVs, with minimal amounts of cellular debris (data not shown). A silver-stained SDS-PAGE gel was used to analyze variations in OMV protein content in each strain. We found that many of the proteins in OMVs isolated from *degS::cat* were different from those in OMVs from the wild-type or complementated strains, and that some of the proteins that were common between strains varied in amount (asterisks, Figure 4B).

degS::cat does not alter antibiotic susceptibility

An antibiotic disk diffusion assay was used to compare the antibiotic susceptibility of the wild-type strain and *degS::cat*. This method was used as a simple way to assess changes in essential pathways (DNA replication, protein synthesis, cell wall synthesis). The assay showed no significant differences in susceptibility to ampicillin, kanamycin, novobiocin, spectinomycin, or tetracycline (p = 0.10, 0.09, 1.0, 0.07 and 0.34, respectively; Figure 5). We therefore conclude that *degS* deletion and the resulting increase in OMV production do not alter antibiotic resistance and that most essential pathways are intact. There was a significant difference in susceptibility to chloramphenicol ($p < 0.000001$, Figure 5). As a resistance cassette for this antibiotic was used to replace *degS* in the mutant, chloramphenicol functioned as our positive control for the assay.

degS::cat bacteria cause less severe infections in a murine cystitis model

Female C57BL/6J mice were infected using a well-established murine model of cystitis in order to compare the virulence of wild-type and mutant strains. At 24 hpi, mice inoculated with *degS::cat* had approximately 100-fold fewer bacteria in the bladder than mice inoculated with UTI89 ($p < 0.0001$, Figure 6).

degS mutation does not alter intracellular growth *in vivo*

A murine model was originally used to characterize bacterial growth during UTI. Growth within bladder epithelial cells and the formation of biofilm-like intracellular bacterial communities (IBCs) are critical if an infection is to progress to high titers. The important role of IBCs was confirmed by the discovery of these structures

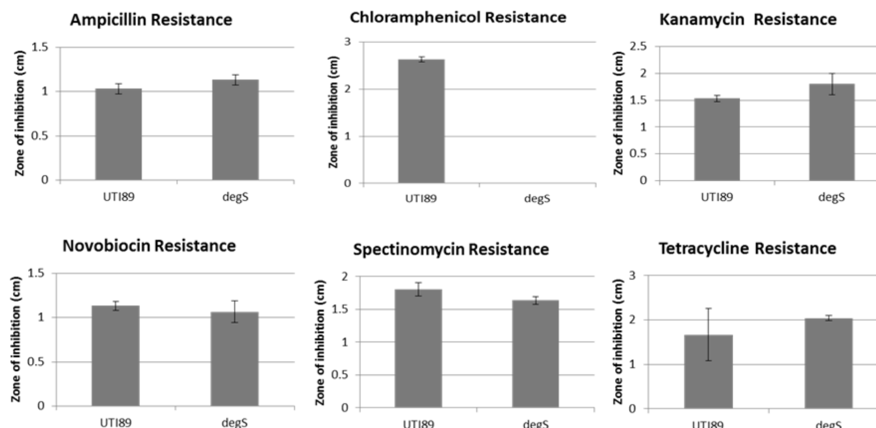

Figure 5: Antibiotic susceptibility. The antibiotic susceptibility of the wild-type and mutant strains was determined by disc diffusion assay. The average zone of inhibition was graphed, error bars represent standard deviation.

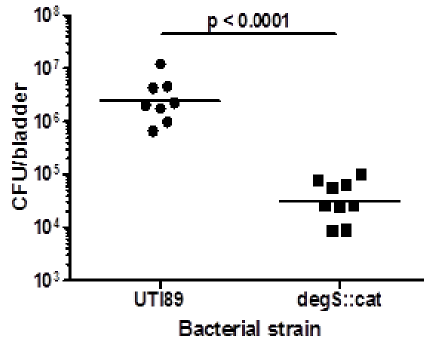

Figure 6: Bladder bacterial titers from infected mice. Colony-forming units (CFU) recovered from mouse bladders at 24 hours post-infection (hpi). Horizontal lines indicate the geometric mean.

in the urine of human patients with active cystitis (15). Knowing this, fluorescent confocal microscopy was used to examine the growth of wild-type and mutant strains within murine bladder epithelial cells to investigate a potential cause for the differences in bladder titers. The bladders of C57BL/6J mice were inoculated with UTI89/pcomGFP or *degS::cat*/pcomGFP. Bladders were harvested at 16 hpi and stained for microscopy. We found both early stage and mature IBCs in the bladders regardless of infecting strain, indicating that both strains were capable of growing within bladder epithelial cells and forming mature IBCs (Figure 7). It should be noted that IBCs were more difficult to identify in mice infected with the mutant strain, as all bacteria seemed to have lost the GFP-expressing plasmid for an unknown reason.

degS::cat has decreased ability to invade host cells

Binding and invasion assays were conducted in order to compare how well the wild-type, *degS::cat*, and p*degS* strains bound and invaded 5637 human bladder epithelial cells *in vitro*. We found that *degS::cat* bound to 5637 cells as well as the wild-type strain (p = 0.47, Figure 8A). However, the *degS::cat* strain invaded cells significantly less than the wild-type strain (p = 0.01, Figure 8B). We found that only 0.1% of the *degS::cat* bacteria that bound to cells actually invaded, as compared to 0.59% of wild-type bacteria (Figure 8C). p*degS* was able to bind and invade as well as the wild-type strain (p = 0.83 and 0.58, respectively, Figure 8A-B). *degS::cat* bound similarly to the complemented strain (p = 0.28, Figure 8A), but invaded significantly less (p = 0.001, Figure 8B). A strain that is unable to produce pili (UTI89 *fimH::kan*) functioned as a binding mutant and our negative control. As expected, UTI89 *fimH::kan* was unable to bind to host cells, and as a result, invaded significantly less than the wild-type strain (p = 0.04, 0.02, respectively; Figure 8A-B).

Discussion

To investigate the role that *degS* and OMVs have on the virulence of UPEC, we characterized a *degS* mutant strain. This mutant has *degS* replaced with a chloramphenicol antibiotic resistance cassette. We predicted that *degS::cat* would be more virulent because of the increased production of OMVs. OMVs have previously been shown to contain outer membrane proteins, toxins, and other factors that can be delivered

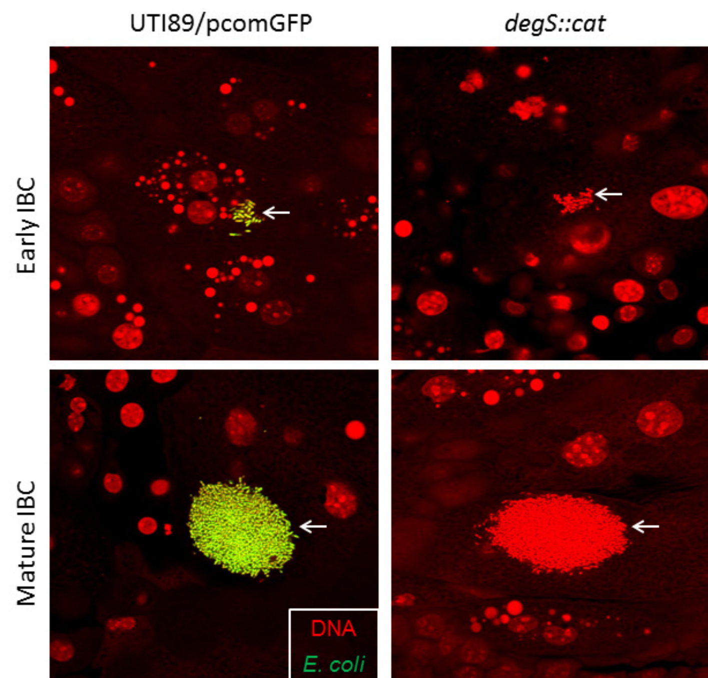

Figure 7: Confocal microscopy images of IBCs. Bladders of mice infected with UTI89/pcomGFP or UTI89 *degS::cat*/pcomGFP were harvested at 16 hpi. IBCs in the bladders were visualized at 63x. Nucleic acids are stained red. IBCs are indicated with an arrow.

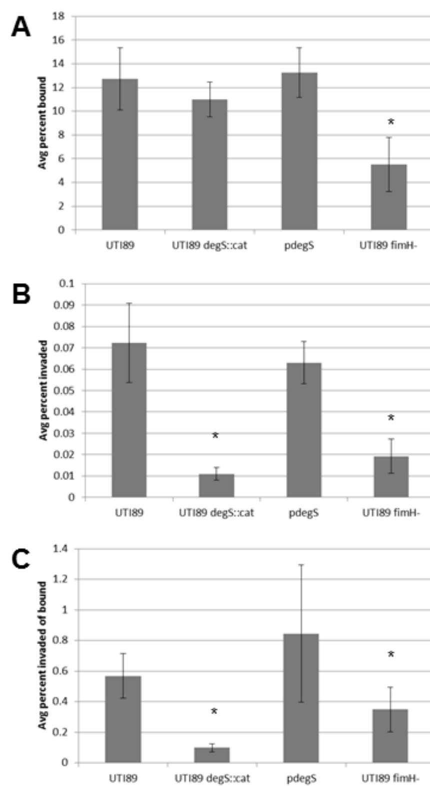

Figure 8: Binding and invasion into 5637 human bladder epithelial cells. (A) The average percent of UTI89, *degS::cat,* p*degS* or UTI89 *fimH::kan* (negative control for binding) cells that were able to bind to host cells. (B) The average percent of each strain that was able to invade into the host cells. (C) The average percent of bound bacteria that invaded into host cells. An asterisk (*) denotes significance as determined by Student t-test compared to wild-type UTI89 ($p < 0.05$). Error bars represent standard deviation.

to host cells, which may in turn enhance infection (9,10). However, our results demonstrated that *degS* may instead be necessary for virulence and that increased numbers of OMVs do not promote infection.

Our experiments with *degS::cat* demonstrated that while *degS* mutation does not alter the morphology of the bacteria under static or shaking growth conditions, the mutant has slightly impaired growth in rich media in early log phase. This difference is no longer significant by mid-log phase, although the mutant does grow at a slightly decreased density. Complementation of the mutation restores the wild-type growth phenotype. These data may indicate that increased OMV production early in growth modifies nutrient allocation and limits the speed at which bacteria can replicate. Similarly, DegS function may be most important in early log phase, when proteases can easily be overwhelmed by misfolded proteins that accumulate as the bacteria rapidly synthesize proteins necessary for growth. In contrast to this idea, the comparison of isolated membranes did not reveal major changes in protein content. However, in the cytosol, a high molecular weight protein was missing in *degS::cat*, which could be related to a change in virulence.

We were able to confirm that *degS* deletion results in increased OMV production, and furthermore, were able to restore wild-type production of OMVs by complementing the mutant with the wild-type gene. Interestingly, in addition to increased numbers of OMVs, *degS::cat* also had changes in the proteins contained within those OMVs. In line with the previous suggestion, these likely represent misfolded proteins that have accumulated in the periplasm due to the lack of functional DegS. Future experiments could confirm this hypothesis by isolating and comparing the periplasm of our three strains. The variability in the protein content of the OMVs and the whole cells likely demonstrates the efficiency for packing misfolded proteins specifically into OMVs to clear them from the cells. We acknowledge that one or more of those proteins could be negatively affecting the virulence of the mutant *in vitro* and/or *in vivo*. The *degS::cat* and wild-type strains also had similar antibiotic susceptibility, indicating that the basic processes through which these antibiotics function are not affected by increased OMV production or by the presence of extra misfolded proteins present in the periplasm due to the decreased DegS activity.

In vitro binding and invasion assays and mouse infections showed that *degS::cat* is less virulent than wild-type. The binding and invasion assays demonstrated that *degS::cat* cells were able to bind to host cells just as well as the wild-type strain, indicating that *degS::cat* does not alter pili production or function. This confirmed our TEM result showing no obvious change in the number of pili produced by the mutant under static conditions. Surprisingly, *degS::cat* was not able to invade the cells. A complemented mutant strain was able to bind and invade as well as the wild-type strain. However, the *degS::cat* strain bound and invaded significantly less than its complement. This confirms that the deletion of *degS* specifically leads to decreased ability to invade. These results may mean that *degS* is important for the invasion mechanism in this model, or that increased production of OMVs is detrimental to mutant cells. Past research has shown that UPEC invade host cells via an HDAC6-modulated microtubule-dependent pathway (16); because *degS::cat* was not able to invade host cells, this may mean that excess numbers of OMVs, or a factor packaged in or on OMVs, can disrupt this pathway. It is also possible that *degS* mutants have limited ability to produce important virulence factors due to energy wasted on misfolded proteins and OMV production.

In a mouse model of cystitis, we found that mice infected with *degS::cat* had 100-fold fewer bacteria in their bladders at 24 hpi. Consistent with our results, past research has also shown that *degS* is necessary for virulence in a murine model of peritonitis and that σ^E regulation is essential for UTI (17, 18). We did not find any differences in intracellular growth of the bacteria when we assessed the bladders for IBC formation. However, IBCs seemed to occur less frequently in mice infected with *degS::cat* (observation by the authors).

Future experiments could quantify IBC formation in mice infected with the wild-type and mutant strains to determine if there is an invasion defect *in vivo* as well. Additional experiments could also confirm that the complemented strain is able to rescue the infection defects.

The function of the DegS protein is relatively well understood. Previous studies have indicated that DegS functions as a protease *in vivo*, contributes to the regulation of σ^E activity, and clears incorrectly assembled proteins out of the periplasm of the cell (4-6). When *degS* is not present, OMV production increases, likely as a result of stress on the cell envelope (7). In our strain, OMV production may be using more of the available energy, which could explain why *degS::cat* grew a little slower than wild-type. Although OMVs have previously been shown to positively impact infection (9, 10), we demonstrate decreased virulence in our UPEC strain when *degS* is knocked out. This suggests that increased OMV production in UPEC could be stressing the cell instead of aiding in infection. Alternatively, the extra vesicles present in mice infected with *degS::cat* could increase the activation of the host immune system, resulting in faster clearance of the mutant bacteria. Finally, our experiments do not conclusively determine whether the altered virulence is a due to greater numbers of OMVs or to a different factor specific to the *degS* mutant. Further research can be done to focus solely on the effects of OMVs in UPEC virulence. Specifically, wild-type UPEC can be tested *in vitro* and *in vivo* with exogenous OMVs to determine whether OMVs alter virulence.

By investigating bacterial virulence factors and important bacterial genes, we can better understand how bacteria function. This can allow chemists to create drugs that are more effective against specific bacteria and help doctors determine how best to treat infections. *E. coli* persists throughout our bodies and our environment, so an in-depth understanding of UPEC virulence could aid in reducing UTI occurrence and recurrence, as well as associated healthcare costs.

Methods

Bacterial Strains

The model uropathogenic *E. coli* strain UTI89 was used as the wild-type. This strain was originally isolated from the urine of a patient with cystitis (11). A *degS* deletion (UTI89 *degS::cat*) was previously made by replacing *degS* with a chloramphenicol (cat) resistance cassette using lambda red mutagenesis (12). A UTI89 *fimH::kan* strain was previously made by replacing the *fimH* gene with a kanamycin (kan) resistance cassette. This strain does not express type 1 pili. All strains were grown in fresh LB broth without antibiotics, unless otherwise indicated.

Molecular Biology

A *degS* complementation plasmid was created by amplifying *degS* from UTI89 genomic DNA (F primer: 5'-CCATCATGTTTGTGAAGCTCTTACGTTCCGTTGC GATTGG-3', R primer: 5'-TTAGTTGGTCGCCGGATAT TCCTGAATGGTGACCTGC-3'). The product and the plasmid vector pTRC99A were digested with XbaI and HindIII and then ligated together. The resulting plasmid was transformed into TOP10 cells. Colony PCR identified colonies that contained the plasmid with the insert, and these colonies were sent for sequencing. Once the sequence was confirmed, the plasmid was electroporated into UTI89 *degS::cat* cells. The full strain is termed UTI89/pTRC99A-*degS degS::cat* and is referred to as p*degS*.

Growth in Rich Media

UTI89, UTI89 *degS::cat* and UTI89 *degS::cat*/p*degS* were cultured in a shaker overnight at 37°C in 3 mL LB broth (with 100 µg/mL ampicillin for p*degS*). The next morning, cells were sub-cultured 1:100 into fresh LB broth with or without antibiotics as appropriate and grown with shaking until the OD_{600}=1 (approximately 2x109 CFU/mL). The cells were diluted to 2x105 cells/mL. Approximately 2000 cells were added to 200 µL of LB per well. Strains were plated in triplicate in LB or LB-Amp. Cells were grown at 37°C, and the optical density at 600nm was recorded every 40 minutes for 12 hr. Triplicate wells of LB and LB-Amp served as blanks and controlled for contamination. The experiment was repeated three times.

Transmission Electron Microscopy (TEM)

UTI89 and UTI89 *degS::cat* cells were grown in LB broth overnight; replicates for each strain were grown under shaking or static conditions. The cultures were centrifuged at 5000 x g for 2 min, and the pellets were resuspended in fresh phosphate-buffered saline (PBS). Samples were then fixed with 1% glutaraldehyde and negative-stained with uranyl acetate before analysis on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) (13).

Membrane Isolation

Overnight cultures of mutant and wild-type strains were sub-cultured and grown in LB broth until they reached an OD_{600} =0.8. Cells were pelleted and the pellets were stored at -20°C. The pellets were resuspended in 10 mM Tris pH 8.0 with 100 mM RNase and DNase. Cells were lysed in the Stansted Pressure Cell Homogenizer (model SPCH-10, United Kingdom). Whole cells were removed by centrifugation at 3000 x g for 15 minutes at 4˚C. The cytosolic and membrane proteins were resuspended in 50 mM Tris. Membranes and cytosol were separated by centrifugation at 33,000 rpm (82,500 x g) for 80 minutes at 4˚C. The pellet containing the membranes was resuspended in 50 mM Tris and centrifuged as before. The supernatant (inner membrane) was removed, and the pellet (outer membrane) was resuspended in 10 mM Tris. All samples were stored at -20°C.

Protein Expression Analysis

SDS-PAGE analysis was used to compare the proteins in whole bacteria, inner membrane, outer

membrane, and cytosol from the wild-type and mutant strains. Samples from each fraction were separated on a 12% SDS-PAGE gel. Gels containing whole cell lysates, outer membranes, and cytosol were Coomassie stained for 30 minutes and then destained with 6:3:1 water:methanol:acetic acid until bands could clearly be seen. To visualize the inner membrane proteins of the wild-type and mutant strain, a Pierce Silver Stain Kit was used according to the manufacturer's instructions (Thermo Scientific).

Bacterial Outer Membrane Vesicle Isolation and Protein Expression Analysis

Wild-type, *degS::cat*, and p*degS* strains were grown in 100 mL LB overnight, before centrifugation for 10 minutes at 5000 x g at 4°C. The supernatant was filter sterilized with 0.2 µm filter and ultracentrifuged for 2 hours at 40,000 rpm (100,000 x g). The pellet was resuspended in 200 µL of Dulbecco's phosphate buffered saline (Gibco) with 0.2 M NaCl. A BCA protein assay was performed according to the manufacturer's instructions (Pierce). The samples were separated by SDS-PAGE and silver stained to compare the proteins in the OMVs.

Antibiotic Susceptibility

UTI89 and UTI89 *degS::cat* were cultured in a shaker overnight at 37°C in 5 mL LB broth. Strains were sub-cultured the next morning and grown to an OD_{600}=1. The cells were diluted 1:10 in sterile PBS. The cultures were then spread evenly across the entire plate with a sterile swab. Using sterile tweezers, three antibiotic-impregnated disks were placed onto each agar surface. A single strain and antibiotic combination was used per plate. The antibiotics for each disk were as follows: ampicillin (10 µg/disk), chloramphenicol (30 µg/disk), kanamycin (30 µg/disk), novobiocin (30 µg/disk), spectinomycin (100 µg/disk), and tetracycline (30 µg/disk). The plates were incubated overnight at 37°C, and the zone of inhibition around the disk was measured. The experiment was repeated three times.

Mouse Infections

C57BL/6J (Jackson Laboratories, Bar Harbor, Maine) mice were used for infections; all mice were female and aged-matched at 8-10 weeks. UTI89 and UTI89 *degS::cat* were grown in 20 mL LB broth overnight under static conditions. Catheters were made with 30 gauge needles and 0.28 mm polyethylene tubing and sterilized with UV light in a tissue culture hood overnight. Overnight cultures were spun down at 7000 rpm for 10 min, resuspended in sterile PBS to OD_{600}=1, and diluted 1:1 with PBS for the inoculum. Under isoflurane anesthesia, three mice per strain were infected transurethrally with 50 µL of bacteria (~1 x 10^7 bacteria). Bladders were harvested 24 hours post-infection (hpi) using sterile technique, homogenized, and plated in order to count colony forming units. The experiment was performed three times.

Confocal Microscopy

Mice were infected with 50 µL of UTI89/pcomGFP or UTI89 *degS::cat*/pcomGFP (2 mice/bacterial strain) as described above. Mice were sacrificed at 16 hpi and bladders were harvested, bisected, and stretched. Bladders were fixed in 2.5% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with Syto 61 nucleic acid stain (1:1000 in PBS, Life Technologies). Stained bladders were washed 3 times with PBS, mounted on slides with ProLong Gold antifade reagent (Invitrogen), and imaged on a Zeiss LSM510 inverted confocal microscope.

Eukaryotic Cell Culture

Human bladder epithelial cells (strain 5637, ATCC HTB-9) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Sigma). Cells were grown at 37°C in a humidified atmosphere with 5% CO2.

In vitro Binding and Invasion Assays

Binding and invasion assays were performed as described previously (14), with modifications. Four strains were compared: UTI89, *degS::cat*, p*degS*, and UTI89 *fimH::kan* (negative control). Overnight static cultures of each strain were centrifuged and resuspended in sterile PBS to OD_{600}=1. Binding, input, and invasion wells with confluent 5637 cells were prepared in triplicate for each strain in 24-well tissue culture plates (TPP). Old medium was aspirated and 1 mL of fresh medium was added to each well. Cells were infected by adding 10 µL of the desired bacterial suspension to each well. Plates were spun at 1500 rpm for 3-5 minutes at room temperature and incubated in a tissue culture incubator for desired length of time. Binding and input wells were removed from the incubator after 30 min. Supernatants were removed from the binding wells, and cells were washed five times with 1X PBS before lysis in 0.1% Triton X-100 (in PBS) at room temperature for 15 min. Input wells were not washed before cells were lysed with 10 µL of 10% Triton X-100. Each well was scraped and examined under the microscope to ensure that all cells were released. Lysed cells and bacteria were serially diluted and plated on LB agar plates to determine CFU. The invasion plates were removed from the incubator after 60 min, and wells were washed once with 1X PBS. Gentamicin (10 µg/mL in media) was added to each well, and the plate was returned to the incubator for 1.5 hours. After gentamicin treatment, wells were washed three times with 1X PBS before lysis in PBS with 0.1% Triton X-100. Lysed cells and bacteria were scraped, serially diluted, and plated. The LB agar plates were incubated overnight at 37˚C and CFU were counted the following day.

Statistical analysis

Significance was determined by a Student t-test, with values less than 0.05 considered significant. For the mouse infections, significance was determined by the Mann-Whitney U statistical analysis as performed by GraphPad Prism 5 software.

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