Phosphoethanolamine Cellulose Enhances Curli-Mediated Adhesion of Uropathogenic *Escherichia coli* to Bladder Epithelial Cells

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SUPPORTING INFORMATION

11 pages with
Figures S1-S3
Table 1
Supporting Video Legends
Supporting Methods
Supporting References

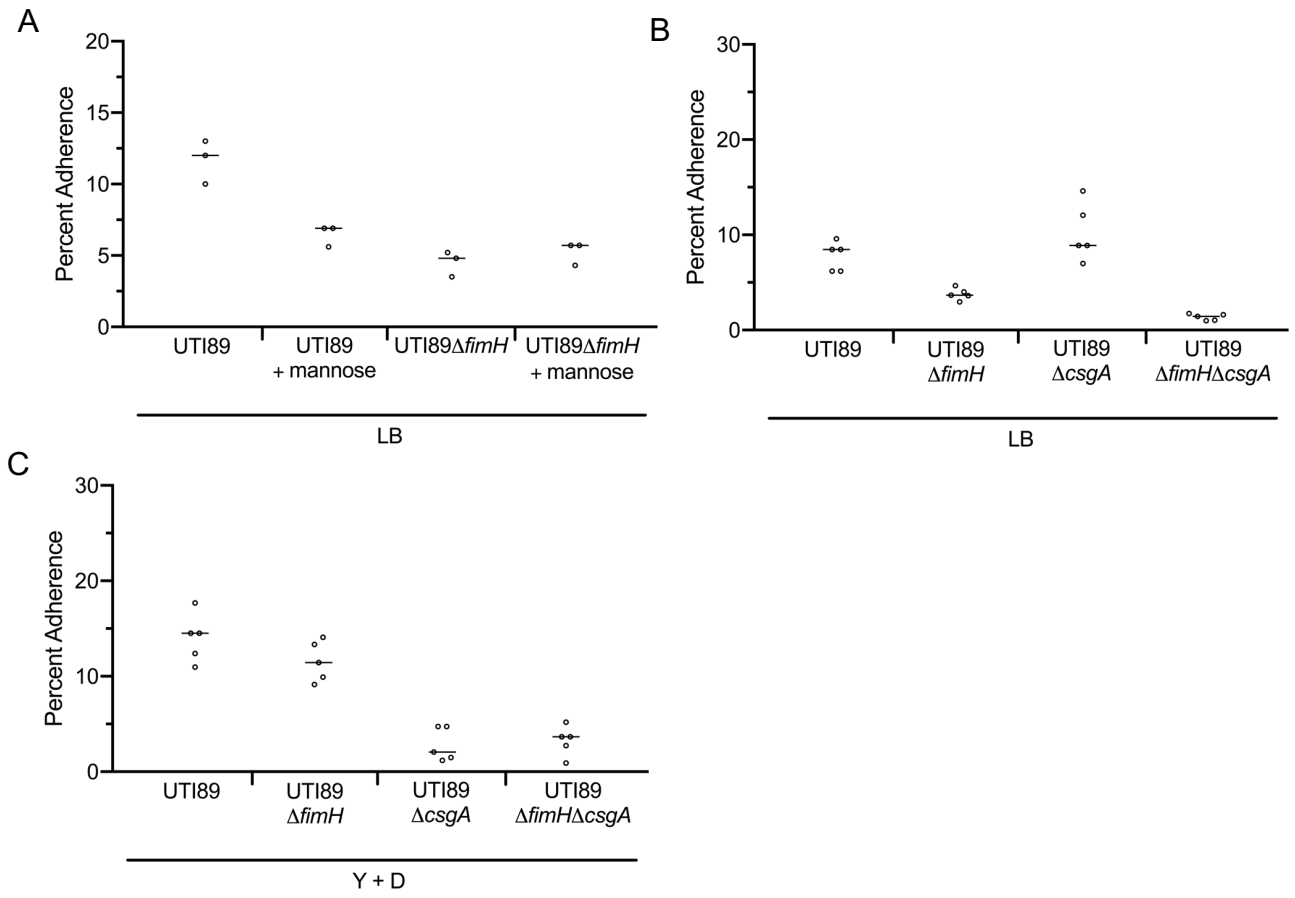

Figure S1. Contribution of curli and type 1 pili to bladder cell adhesion in traditional adhesion assays. (A) Adhesion assay results are provided as percent adherence for adhesion assays comparing UTI89 and its isogenic mutant unable to produce the type 1 pilus adhesin FimH (UTI89Δ*fimH*), both with and without 2.5 % (w/v) α-methyl-D-mannopyranoside (a FimH receptor analog) added to the bacterial culture. (B) Adhesion assay results are provided as percent adherence to compare UTI89, UTI89Δ*fimH*, UTI89Δ*csgA,* and UTI89Δ*fimH*Δ*csgA* grown under conditions promoting production of type 1 pili. (C) Adhesion assay results are provided as percent adherence to compare UTI89, UTI89Δ*fimH*, UTI89Δ*csgA,* and UTI89Δ*fimH*Δ*csgA* grown under conditions promoting production of curli. Each point represents the result from one well in the plate and the bar represents the median of each data set. Bacterial growth conditions: LB broth, 37˚C, static culture (A and B), YESCA broth + 4% DMSO, 26˚C, shaking culture (C).

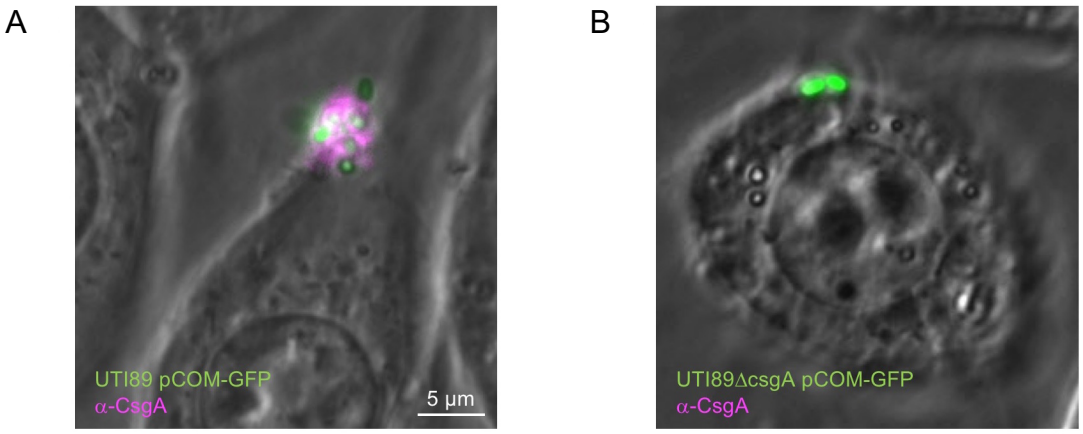

Figure S2. Interbacterial interactions in the traditional adhesion assay. (A) UTI89 transformed with a plasmid that constitutively expresses GFP (pCOM-GFP) adheres to a bladder cell in a cluster when expressing curli. (B) The isogenic mutant unable to produce curli, UTI89Δ*csgA*, is not clustered and instead adheres as individual bacteria.

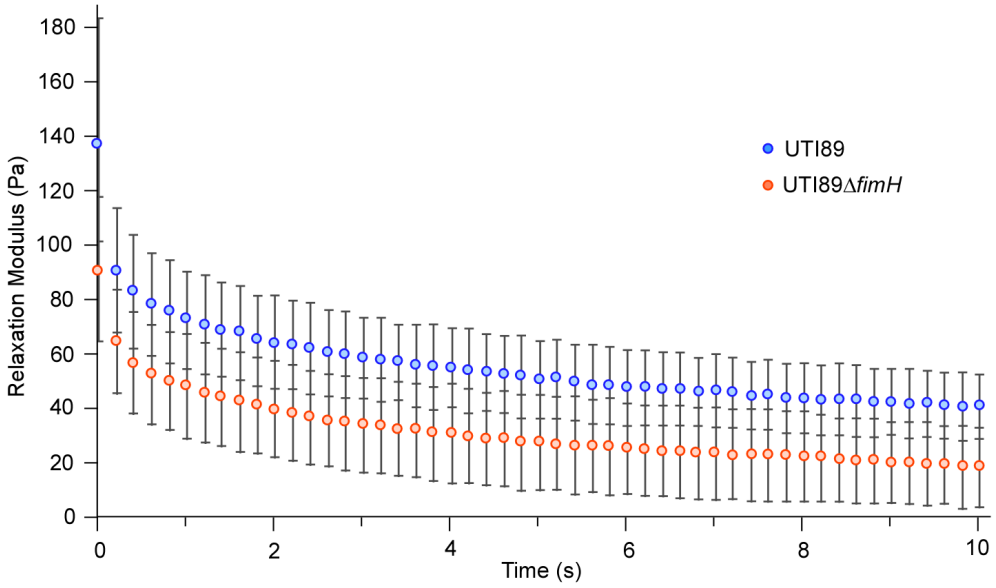

Figure S3. Comparison of UTI89 and UTI89Δ*fimH* adhesion to bladder epithelial cells in the LCMR. Relaxation moduli over time for LCMR experiments comparing the adhesiveness of UTI89 producing type 1 pili and UTI89 lacking the pilus adhesion, UTI89Δ*fimH*, both grown in LB conditions to promote type 1 pilus expression. UTI89Δ*fimH* exhibits reduced adhesion in the LCMR experiment.

Table 1. Primers used for generating and verifying a chromosomal deletion of *csgA*.

csgA_R	ATGAAACTTTTAAAAGTAGCAGCAATTGCAGCAATCGTATTCTCTGGTA GCATATGAATATCCTCCTTAG
csgA_L	TTAGTACTGATGAGCGGTCGCGTTGTTACCAAAGCCAACCTGAGTGACG TGTGTAGGCTGGAGCTGCTTC
csgA30d	GCGCCCTGTTTCTGTAATAC
csgA20u	CAATCCGATGGGGGTTTTAC

Supporting Video Legends

Video S1: Shearing of fibronectin-coated top plate across bladder cell monolayer. LCMR experiment in which the top plate is coated in only fibronectin (control case). The bladder cells are not significantly deformed by the motion of the top plate, indicating low adhesion between the fibronectin coating and bladder cells.

Video S2: Shearing of curli-coated top plate across bladder cell monolayer. LCMR experiment in which the top plate is coated with curli. Curli are attached to the top plate via a fibronectin coating. The bladder cells are visually deformed by the motion of the top plate, indicating strong adhesive interactions between the curli fibers and bladder cells.

Supporting Methods

Bacterial Strains and Culture Conditions. Strains used in this study include uropathogenic strain UTI89 and mutants UTI89Δ*csgA*, UTI89Δ*bcsA,* UTI89Δ*fimH*, and UTI89Δ*csgA*Δ*fimH*. UTI89 Δ*csgA*Δ*fimH* was constructed for this study by a chromosomal deletion of *csgA* gene in strain UTI89Δ*fimH* using a method described in Datsenko and Wanner.[1] The FRT-flanked resistance gene was produced by PCR using a pKD4 template and csgA_R and csgA_L primers (Table 1) as reported previously in the construction of UTI89Δ*csgA*.[2] The deletion of the *csgA* gene was confirmed by PCR using *csgA*-flanking primers, csgA30d and csgA20u (Table 1). The kanamycin cassette was removed by introduction and expression of an FLP recombinase-expressing plasmid pCp20. The temperature sensitive pCp20 plasmid was subsequently eliminated by passaging at 42 ˚C. Strains used in this study include uropathogenic strain UTI89 and mutants UTI89Δ*csgA* and UTI89Δ*bcsA*. Bacterial stock cultures were prepared on LB agar and single colonies were inoculated to either YESCA medium (yeast extract 1 g/L, casamino acids 10 g/L) or LB medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L). To promote moderate curli production, bacteria were grown in YESCA medium supplemented with 4% DMSO at 26˚C and shaking at 200 rpm. To promote type 1 pili production, bacteria were grown in LB medium at 37˚C without shaking. For both conditions, cultures were diluted 1:1000 after 24 h of growth, and grown for another 24 h.

Mammalian Cell Culture. The human bladder epithelial cell line 5637 (ATCC HTB-9) was maintained in RPMI 1640 medium (GE Healthcare) supplemented with 10% fetal bovine serum (Corning) and Penicillin-Streptomycin (MP Biomedicals) at 37˚C with 5% CO_2.

Traditional Cell Adhesion Assay. Bladder cells were seeded into 24-well tissue culture plates and grown for 18-24 hours to confluency. Prior to the assay, the medium was changed from RPMI 1640 medium to CO_2-Independent medium (Gibco) by washing the cell monolayers once with phosphate buffered saline (PBS, pH=7.4, -CaCl, -MgCl) (Gibco), then adding 1 mL of CO_2-Independent medium to each well. Bacteria were added to each well at a multiplicity of infection of 15. Plates were centrifuged at 600g for 5 minutes and allowed to sit for 10 min at room temperature. Each well was then washed 5 times with PBS to remove non-adherent bacteria, the cell monolayer was disrupted with 0.5% Triton X-100 (Sigma Aldrich), and the remaining adherent bacteria were enumerated. Experiments performed on the same set of bladder cells are presented together.

Curli Purification. The purification of curli was based on previously described protocols[3,4] using the curli-producing strain MC4100. The final concentration of curli in Tris buffer was checked using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit), then adjusted to 1 mg/mL using Tris buffer. Curli were extracted and purified the day before each experiment.

Live Cell Monolayer Rheometer (LCMR). The basic design and implementation of the LCMR has been described previously[5,6]. Briefly, the instrument consisted of a custom-built bottom plate on which bladder cells were cultured. This bottom plate was mounted on an inverted microscope (Nikon Eclipse T*i*-S, Nikon) and cells were visualized using differential interference contrast microscopy and an 100x oil-immersion objective (Plan Apo VC 100X Oil, Nikon) which was maintained at 37˚C via an objective heater (Bioptechs Objective Heater System, Bioptechs). A custom-built top plate was brought into contact with the cell monolayer. This applied a constant normal stress to the cells of approximately 75 Pa and was the same for all experiments. This normal stress ensured good contact between the cell monolayer and top plate without overly deforming the cells, *e.g.*, inducing blebbing or cell lysis. The top plate consisted of a plano-convex lens (KPX124, effective focal length 1000 mm, 1-inch diameter, Newport). The planar side of the lens was shaved down to give an average lens thickness of ~250 μm and was attached

to an aluminum mount. The convex side of the lens was in contact with the cell monolayer. The use of the curved lens surface facilitated close proximity (on the order of a bladder cell body) of the top and bottom plates and avoided the possibility of direct contact at the edges. The selection of a long distance focal length lens ensured that the gap between the plates varied no more that 5% over the contact area. A force transducer (400A, Aurora Scientific) attached to a piezoelectric stage (M-011.PS, Physik Instrumente) was brought into contact with the aluminum mount of the top plate. Lateral motion of the piezoelectric stage and attached force transducer pushed the top plate along the cell monolayer, shearing the cells.

Bottom Plate Preparation. A glass coverslip (#2, 18x18 mm) was coated with collagen (type 1, rat tail, Corning) at 5 $\mu g/cm^2$ for 1 hour at room temperature. Excess collagen solution was removed, and the coverslip was rinsed twice in PBS. The coverslip was then sealed into a custom-made aluminum plate using UV curing adhesive (Norland Optical Adhesive 61, Thorlabs) and silicone grease (Dow Corning). The assembled bottom plate was sprayed with 70% ethanol and left overnight in a tissue culture hood with the UV sterilization light turned on. In the morning, bladder cells were detached from their culture flask and suspended in RPMI 1640 medium, counted using a hemocytometer, adjusted to a density of ~$3x10^6$ cell/mL, and 600 µL of the cell suspension was deposited onto the glass coverslip of the bottom plate. After incubation for 2 hours at 37˚C and 5% CO_2, which gave the cells time to attach to the glass coverslip, 3.5 mL of RPMI 1640 medium was added to the bottom plate for further incubation. After overnight incubation, the cells formed a confluent monolayer on the glass coverslip. Before the LCMR experiment, the cell monolayer was washed twice with PBS and 4 mL of CO_2-Independent medium was added to the bottom plate.

Top Plate Preparation. As the custom-made top plate was reused for each experiment, cleaning of the top plate was achieved by separating the glass piece from the aluminum mount by soaking in acetone. The glass was then wiped with a lens cloth soaked in 70% isopropanol and cleaned using oxygen plasma (Plasma Prep 5, Gala Instrumente) for 10 minutes.

If the glass was to be treated with purified curli fibers, it was then coated with fibronectin (F1141, Sigma Aldrich) at 5 $\mu g/cm^2$ for 1 hour at room temperature. Excess fibronectin solution was removed, and the glass was rinsed twice with MilliQ water. Next, 500 µL of a 0.0005 wt% solution of 1 µm polystyrene beads (ThermoFisher Scientific) in MilliQ water was allowed to

air-dry onto the glass, leaving behind a sparse coating of the beads. Finally, 500 μL of a 1 mg/mL solution of purified curli (see section "Curli Purification") was added to the glass and left overnight, allowing the curli fibers to absorb. Prior to the LCMR experiment, excess curli was rinsed from the glass with PBS and the glass was reattached to the aluminum mount using UV cured adhesive. Curli fibers remained hydrated at all times from initial purification to the end of the experiment.

If the glass was to instead be covered in a covalently linked layer of bacteria, after oxygen plasma cleaning, the glass was placed in a 6-well plate and 200 μL of poly-L-lysine solution (P4707, Sigma Aldrich) was allowed to air-dry onto the glass. Next, 500 μL of a 0.0005 wt% solution of 1 μm beads was allowed to air-dry onto the glass. The glass was then treated with 500 μL of 2.5% glutaraldehyde (Fisher Chemical) in PBS for 1 hour at room temperature. Excess glutaraldehyde solution was removed, and the glass was rinsed twice with PBS. 5 mL of bacteria suspended in PBS was added to the well containing the glass and the 6-well plate was centrifuged at 2,250g for 5 minutes to bring the bacteria into contact with the glass. The plate was then left to sit for 1 hour at room temperature. Prior to the LCMR experiment, excess bacteria were rinsed from the glass with PBS and the glass was reattached to the aluminum mount using UV cured adhesive. The bacteria remained hydrated at all times during the attachment process to the end of the experiment.

Step-Strain Experiment. A bottom plate containing a monolayer of bladder cells and 4 mL of CO_2-Independent medium was placed on the inverted microscope and brought into contact with the heated 100X objective. Next, the top plate, coated with either curli or bacteria, was slowly lowered through the medium onto the cell monolayer using a manual miniature translation stage (M-105.10, Physik Instrumente). Contact between the top plate and cell monolayer was confirmed by a visual expansion of the cell area due to slight pressure from the top plate. At this point, an additional 2 mL of CO_2-Independent medium was added to the bottom plate. After a period of 15 minutes, the gap distance between the top plate and bottom plate was determined from the focal adjustment between the two plates; the gap distance in microns (d) was calculated using a known conversion factor. A force transducer attached to a piezoelectric stage was then gently brought into contact with the aluminum mount on the top plate. To perform a step-strain, the piezoelectric stage was programmed to push the top plate along the cell monolayer a

specified distance and hold the final position. During and after the top plate motion, a custom LabView code recorded the applied force (F) measured by the force transducer and a CCD camera (GuppyPro F-125 B, Allied Vision) connected to the microscope recorded the movement of the top plate and deformation of the cells. Image analysis of the pixel positions of the 1 μm beads on the top plate before and after the step gave the top plate lateral displacement; the top plate displacement in microns (l) was calculated using a known conversion factor. Note that the beads remained in focus during and after the step, indicating the top plate remained level during the experiment. After the initial step-strain motion, the force was recorded for 2 minutes, then the force transducer was retracted and was no longer in contact with the top plate. After a waiting period of 10 minutes, allowing the system to return to its initial state, the force transducer was again gently brought into contact with the top plate and the next step-strain experiment was performed. Step-strain experiments were performed in order of increasing amount of strain, with each step executed at a nominal strain rate of 20 s^{-1}.

Calculation of Relaxation Modulus $G_r(t)$. To calculate the stress, σ, the force measured by the force transducer (F) was divided by the contact area between the top plate and the cell monolayer (A), which was estimated previously[38]. Thus, the stress as a function of time was calculated by $\sigma(t) = F(t)/A$. The strain, γ, was calculated from the lateral displacement of the top plate (l) and the gap distance between the top and bottom plate (d), *i.e.*, $\gamma = l/d$. The shear relaxation modulus was then calculated as $G_r(t) = \sigma(t)/\gamma$. In all figures, the maximum relaxation modulus, which occurs just after the step-strain motion, has been shifted to time zero. Data is represented as an average of three independent trials; each trial was performed with a new monolayer of bladder cells and a freshly prepared coated top plate.

Immunofluorescent Imaging. Bacteria were grown in YESCA medium supplemented with 4% DMSO, 26˚C, 200 rpm, 48 hrs total growth with 1:1000 dilution after first 24 hours, then pelleted by centrifugation at 10,000g for 5 minutes, and resuspended in sterile MilliQ water. The suspension was allowed to air-dry onto a glass microscope slide, then heat fixed using a Bunsen burner. Slides were blocked for 30 minutes at room temperature with blocking buffer (1% bovine serum albumin in PBS), followed by immunofluorescent staining. The polyclonal rabbit antiserum to CsgA was used as the primary antibody and an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes) was used as the secondary antibody. Slides were also

incubated with Calcofluor White (0.01 mg/mL in PBS) for 10 minutes. Coverslips were mounted with Fluoromount-G (SouthernBiotech) and images were captured using a Zeiss AxioObserver Z1 microscope equipped with a CoolSNAP HQ2 CCD camera (Photometrics).

Transmission Electron Microscopy (TEM). Bacteria were grown under the conditions described above and were then diluted with PBS to OD_{600} 1.0 and applied to the TEM grid (300-mesh copper grids, formvar carbon film, Electron Microscopy Sciences). For the micrographs of vortexed cells, samples were prepared by centrifuging the appropriate volume of the bacterial culture (to give OD_{600} 1.0 when resuspended to 1 mL) at 10,000g for 10 minutes, aspirating the supernatant, resuspending the cells in 1 mL of 10mM Tris buffer (pH=7.4), and vortexing at 1500 rpm on a Digital Vortex Mixer (Fisher Scientific) for 0 or 5 minutes. Cells were applied to the TEM grid and allowed to adsorb for 2 minutes. Grids were washed 5 times with MilliQ water and negative stained with 2% uranyl acetate (Electron Microscopy Sciences) for 90 seconds. After removing the uranyl acetate, the grids were allowed to air-dry for 10 minutes, then examined using a JEM1400 transmission electron microscope (JEOL).

Western Blot Analysis. Bacteria were grown in LB, YESCA, or YESCA medium supplemented with 4% DMSO under the conditions described in the section "Bacterial Culture." Samples were prepared by centrifuging the appropriate volume of the bacterial culture (to give OD_{600} 1.0 when resuspended to 1 mL) at 10,000g for 10 minutes to pellet the cells and aspirate the supernatant. For vortexed samples, pellets were resuspended in 1 mL of 10mM Tris buffer (pH=7.4), vortexed at 1,500 rpm on a Digital Vortex Mixer (Fisher Scientific) for 10 seconds, 1 minute, or 5 minutes, and cells were pelleted again at 10,000g for 10 minutes. The supernatant was saved separately, flash frozen in liquid nitrogen, and lyophilized. All samples were treated with 100 µL of >98% formic acid to dissociate CsgA. Formic acid was removed with vacuum centrifugation, and samples were resuspended in 200 µL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were heated at 95˚C for 10 minutes, allowed to cool, and protein gel electrophoresis was carried out using 12% SDS-PAGE gels (NuPAGE). Proteins were transferred onto 0.2 µm nitrocellulose transfer membranes (GE Healthcare) at 47V and 4˚C for 3 hours, then blocked overnight at 4˚C. The polyclonal rabbit antiserum to CsgA was used as the primary antibody, and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Pierce) was used as the secondary antibody.

Supporting References

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