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Distinct morphological fates of uropathogenic *E. coli* intracellular bacterial communities: dependency on urine composition and pH

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10   Running Title: UPEC morphological responses during cellular infection

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Abstract

Uropathogenic *E. coli* (UPEC) is the leading cause of urinary tract infections (UTI). These bacteria undertake a multi-stage infection cycle involving invasion of and proliferation within urinary tract epithelial cells, leading to the rupture of the host cell and dispersal of the bacteria, some of which have a highly filamentous morphology. Here we established a microfluidics-based model of UPEC infection of immortalized human bladder epithelial cells that recapitulates the main stages of bacterial morphological changes during the acute infection cycle *in vivo* and allows the development and fate of individual cells to be monitored in real-time by fluorescence microscopy. The UPEC-infected bladder cells remained alive and mobile in non-confluent monolayers during the development of intracellular bacterial communities (IBCs). Switching from a flow of growth medium to human urine resulted in immobilization of both uninfected and infected bladder cells. IBCs continued to develop and then released many highly filamentous bacteria via an extrusion-like process, whereas others showed strong UPEC proliferation yet no detected filamentation. The filamentation response was dependent on the weak acidity of human urine and required component(s) in a low molecular-mass (<3000 Da) fraction from a mildly dehydrated donor. The developmental fate for bacteria therefore appears to be controlled by multiple factors that act at the level of the whole IBC, suggesting that variable local environments or stochastic differentiation pathways influence IBC developmental fates during infection.

Introduction

36 Uropathogenic *Escherichia coli* (UPEC) is responsible for more than 80% of urinary tract

37 infections (UTI), which are one of the most common infections that are worsening rapidly as a

38 consequence of pandemic multi-resistant strains spreading globally (1). These invasive infections

39 proceed through a multi-stage intracellular cycle, beginning with the attachment of bacteria to the

40 host urinary tract epithelial surfaces (2, 3). The bacteria are taken up by endocytosis and some are

41 released into the cytoplasm and grow to form tightly packed biofilm-like colonies called

42 intracellular bacterial communities (IBCs) (4-8). Eventually the epithelial cell becomes

43 overwhelmed by bacterial growth, and ruptures, dispersing bacteria that may infect neighboring

44 host cells (9). This cycle amplifies the infection and leads to widespread damage and shedding of

45 layers of the epithelium, leading to the acute symptoms observed in UTI.

46 UPEC show remarkable morphological variation during the infection cycle (10). Whereas UPEC

47 appear as typical rod-shaped bacteria in laboratory cultures, during the infection cycle many of the

48 intracellular bacteria exist as very short rods or almost-spherical smaller cells within well-

49 established IBCs. However, while bacteria are released during IBC dispersal and host cell rupture,

50 UPEC subpopulations appear as rods, motile-rods, and viable highly filamentous bacteria (11).

51 Filamentation occurs by bacterial cell growth (elongation) and ongoing chromosome replication

52 without division, and is a response to certain conditions or stresses (10). Filamentation is a

53 characteristic response of UPEC during UTI and is thought to aid in dispersal and surface

54 attachment after release from the host cell, and might help prevent phagocytosis by immune cells

55 (11-13). It has been demonstrated that UPEC filaments are capable of reverting back into rod-

56 shaped cells, through numerous cell division events along the length of the filaments, that are then

57 capable of infecting other host cells (14).

58 Many of the features of the UPEC infection cycle outlined above were identified with the mouse

59 model of UTI, involving transurethral injection of UPEC into the bladder, and analysis of collected

60 urine/tissues or monitoring of infected bladder explants over time (4, 7, 9). IBCs and filamentous

61 bacteria are also common in human cystitis (8). Cell culture models were more recently developed

62 to allow further analyses of bacterial physiology during infection, based on UPEC infection of

63 human bladder epithelial cell (BEC) surface culture and incubation with growth medium or human

64 urine (14, 15). A static infection model was developed that could be performed in multi-well

65 culture plates, and was used to study features of the early stages of infection and quantify

66   intracellular growth of UPEC within BECs (15). This was not suitable for analysis of the later

67   stages of host cell rupture (bacterial dispersal and re-infection) and a flow-chamber model was

68   then developed, incorporating steps that allowed the analysis of later infection stages (14). This

69   model involved UPEC infection of BECs in flow chambers that were maintained with a constant

70   flow of medium during the early development of IBCs (day 1), and then switched to a flow of

71   filtered human urine, which induced the dispersal/filamentation stage (day 2). Extensive bacterial

72   filamentation was observed on the surface and dispersed into the effluent (14). Given that UPEC

73   show a complex pattern of morphological changes during the infection cycle, and the infection

74   cycle in the population is likely to show a low degree of synchrony, the ability to use time-lapse

75   microscopy to investigate the timing and transitions occurring at the single cell level is expected

76   to be of significant benefit in understanding the sequence of morphological events occurring during

77   the UPEC lifecycle.

78   Here we have established a microfluidics-based cell culture infection model for UPEC infection

79   of immortalized human bladder epithelial cells, combined with a new robust GFP-based bacterial

80   labelling for real-time fluorescence microscopy observation of infections at high resolution. The

81   main advantages of using microfluidics are the use of greatly reduced quantities of reagents and

82   laboratory animals, a high degree of control of conditions, and the ability to conduct multiple real-

83   time infections experiments simultaneously whilst visualizing infection progression and viability

84   of individual cells. This approach has revealed details of how infected cultured cells behave during

85   IBC development, that there are at least two distinct morphological fates of IBCs upon dispersal,

86   and how filaments appear to extrude from infected bladder cells during dispersal. We also

87   demonstrate that multiple conditions including a urinary pH of ~5 are necessary to elicit the UPEC

88   filamentation response during the IBC dispersal and host cell rupture stage of infection.

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Results

Construction of fluorescent derivatives of uropathogenic E. coli UTI89

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91 We first aimed to establish a system for robust fluorescent labelling of live bacteria, to allow their

92 visualization during the course of an intracellular infection. Lambda-red recombination (16) was

93 initially used to replace the *lacZ* open reading frame on the chromosome of model UTI strain *E.*

94 *coli* UTI89 with fluorescent proteins, GFP or mCherry, linked to a downstream Km^R marker;

95 however, fluorescence in the resulting bacteria was too faint to provide a useful label for bacteria

96 in an infection (data not shown). Similar results were obtained with attempts involving

97 transformation of UTI89 with GFP-expression plasmid pXG10 (17), whereas pEGFP-based

98 plasmids reported previously for this purpose (14) gave stronger expression, but required IPTG-

99 induction and showed a variegated fluorescence response between individual bacteria, particularly

100 in the stationary phase of batch cultures (data not shown). A new low-copy number plasmid was

101 then constructed (pGI5), containing the pSC101 origin of replication and the *PlacI^{Q1}* strong

102 constitutive promoter (18) driving expression of a monomeric super-folder GFP (msfGFP) (19).

103 This provided bright, robust and uniform cytoplasmic fluorescent labelling of UTI89 (Fig. S1).

104 Equivalent plasmids based on the significantly weaker *PlacI* and *PlacI^Q* promoters were also

105 constructed (pGI3 and pGI4, respectively), although the fluorescence of UTI89 harboring these

106 was considered too faint for use in most infections (Fig. S1).

A microfluidic infection model involving intracellular growth and dispersal of UPEC

108 We sought to visualize the main infection stages of UPEC intracellular growth and release from

109 BECs using a microfluidics-based culture system, based on the mid-scale flow-chamber model

110 previously established (14). The microfluidics system used here (CellASIC Onix) has a thin glass

111 base plate (170 micron), adhered to a cast set of microfluidic channels supplied by inlet wells filled

112 with the different media needed for each stage of the infection (Fig. 1, upper panel). These wells

113 have microfluidic channels connected to a main culture chamber, where the immortalized human

114 bladder epithelial cells (BECs) were initially grown. The flow of liquid from the wells through the

115 culture chamber and into a waste reservoir is controlled by a manifold and air pressure controller

116 wells through the culture chamber and into waste wells; each chamber can therefore be switched

117 between the flow of different reservoir liquids (*e.g.* growth medium or urine). Four such chamber

118 systems exist per plate (standard microtiter footprint), allowing multiple infections to be observed

119 simultaneously in an incubated inverted microscope with automated stage and focusing.

120   A layer of PD07i BECs, initially grown for two days in the microfluidic culture chambers, was

121   exposed to a capillary-driven flow of *E. coli* UTI89 (+pGI5) for 20 min to allow bacterial

122   attachment, and then the flow was switched to the BEC growth medium (EpiLife) for 9 h to allow

123   bacterial uptake and growth. The flow was then switched to medium containing the BEC-

124   impermeable antibiotic Gentamicin, for 20 h, to kill and remove only the extracellular bacteria and

125   allow IBC development and visualization. Finally, the flow was switched to human urine for a

126   further 20 h to observe bacterial dispersal and host cell rupture. Microscope observation of the

127   cells after the intracellular growth stage (*i.e.* at 29 h post-infection) showed densely packed discrete

128   IBCs within BECs (Fig. 1, lower left) that often appeared near the edge of the nucleus or as multi-

129   lobed colonies. The proportion of infected BECs was typically 2-10% of the total, consistent with

130   previously observed rates of infection *in vitro* (14). At the 49 h stage—after the 20 h exposure to

131   a flow of urine—many highly filamentous bacteria were evident amongst the BECs (Fig. 1, lower

132   right).

133   ***UPEC-infected PD07i bladder epithelial cells retain surface migration capacity***

134   We then observed infections during the intracellular growth stage by recording time-lapse movies

135   of the infections in the microfluidic culture chambers (*i.e.* during the flow of growth medium with

136   Gentamicin, 9–29 h post-infection). As shown in Fig. 2 (and in Movie S1), the bladder cells

137   showed active migration on the surface of the culture chamber during this period. Infected BECs,

138   as indicated by the presence of fluorescent IBCs, moved on the surface in a manner

139   indistinguishable from the uninfected bladder cells in the same field of view, even when containing

140   very large IBCs (Fig. 2, blue arrowhead). Some infected bladder cells were observed to move in

141   or out of the field of view during the time-lapse (Fig. 2, blue and white arrowheads; see also Movie

142   S1). During these experiments, the live-cell-impermeant DNA stain SYTOX Orange was included

143   in the medium (at 100 nM), with the aim of specifically staining and detecting permeable (dead)

144   cells over the course of the infection (Fig. 2, red arrowheads), whilst simultaneously allowing the

145   GFP-labelled bacteria to be located with green fluorescence. SYTOX Orange stained BECs were

146   occasionally seen that showed no evidence of infection, exhibited a rounded shape, and were

147   partially or fully detached from the surface, typical of dead cells (Fig. 2, red arrowheads). In

148   contrast, the surface-bound migratory bladder cells in the monolayer, both infected and uninfected,

149   did not stain with SYTOX Orange (Fig. 2).

150   ***PD07i cells cease surface migration and gain autofluorescence after urine exposure***

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151   The viability and normal surface migration behavior of infected BECs (Fig. 2) clearly showed that

152   IBCs did not globally incapacitate nor substantially affect the BECs during the early stages (first

153   29 h) of IBC establishment and growth. We then switched to a flow of filtered human urine,

154   observing cells 20 min before the switch to urine, immediately after the switch to a flow of urine

155   (at 29 h), and then 10 h into the urine exposure (Fig. 3). The lack of SYTOX Orange staining of

156   BECs before the urine exposure indicated that most BECs were non-permeable, apart from the

157   occasional dead cell (*e.g.* Fig. 3; very bright drifting cells (out of focus), and some stationary ones

158   indicated by white arrowheads). Upon commencement of urine exposure, the background

159   fluorescence in the entire field of view increased significantly in the GFP channel. The brightly

160   labelled intracellular UTI89/pGI5 bacteria were still detectable (*e.g.* Fig. 3, cyan arrowheads).

161   After 10 h of urine exposure, the morphology of the BECs and their location in the field of view

162   had remained unchanged, indicating that the urine treatment immobilized the BECs (Fig. 3,

163   compare phase-contrast images). By 10 h, the BECs also showed much greater whole-cell

164   autofluorescence in the red and especially the green channels (Fig. 3).

165   The above observations can be seen in greater temporal resolution in Movie S2, which shows time-

166   lapse images recorded at 10 min intervals over the 20 h urine exposure (*i.e.* 29 – 49 h post-

167   infection). Cells ceased surface migration immediately upon urine exposure. Then, during the first

168   ~6 h of urine flow, we observed the fluorescence of individual BECs (infected and uninfected) to

169   become brighter at times that varied for individual cells, especially in the green channel. The

170   transition for individual cells to the high autofluorescence state took ~10-40 min. This suggested

171   a permeabilization or metabolic change in BECs in response to the urine. However,

172   permeabilization was difficult to directly assess, as the SYTOX Orange (added to the urine) was

173   not effectively reporting permeabilization in the urine conditions. We observed that the dead BECs

174   that had been brightly stained in the preceding Epilife+Gm stage had lost their bright red

175   fluorescence during the first 10-20 min of urine exposure (Fig 3, white arrowheads; Movie S2).

176   This could be due to either a lack of dye fluorescence/DNA-binding in urine, or the DNA was lost

177   (degraded) in dead cells upon urine exposure. In support of the latter, some bacteria retained both

178   green and red fluorescence during the 20 h urine exposure, the red fluorescence of dead BECs took

179   ~20 min to completely disappear after switching to urine (Movie S2), and human urine contains

180   DNases (20, 21).

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181 *UPEC emerge from infected PD07i bladder epithelial cells via at least two distinct IBC dispersal*
182 *types*

183 Bacteria within an IBC have been reported to grow from short rod-shaped cells into long filaments
184 after exposure to high specific-gravity (SG) urine during the dispersal phase on infection (14). To
185 directly visualize this stage of infection over time, phase-contrast and GFP-fluorescence images
186 were recorded at 10 min intervals during the 25-49 h post-infection period. This 24 h movie
187 included the last 4 h of the growth medium flow conditions, followed by 20 h of urine flow
188 conditions. Seven of the ten IBC dispersal events (individual BEC eruptions) that were monitored
189 (in three flow separate infection channels with the same urine) resulted in the emergence of
190 filamentous bacteria, and three resulted in emergence of rod-shaped bacteria. An example of an
191 IBC that released filaments may be seen in Fig. 4 and Movie S3; infected BECs became overtaken
192 by proliferation of an IBC, followed by the expulsion of filamentous bacteria via an extrusion-like
193 process. The filamentous bacteria began to emerge 6-8 h after urine exposure in this cell, appearing
194 initially as several filaments that grew out from the surface of the BEC, followed by a massive
195 eruption of filamentous bacteria at ~15 h after urine exposure (Fig. 4). Fig. 5 shows another
196 infected BEC with a growing IBC where no filaments were detected. The infected BEC became
197 overwhelmed by the rapidly growing IBC, after a period of moderate development ~5–7 h after
198 urine exposure (Fig. 5). Some bacteria then emerged at around 8 h after urine exposure—possibly
199 motile bacteria—followed soon after by eruption of the IBC and release of a great number of rod-
200 shaped bacteria (Fig. 5). After 13 h of urine exposure (Fig. 5), the bacteria from the ruptured BEC
201 had occupied the entire field of view (Movie S4).

202 *Neutralized and acidified human urine do not induce UPEC filamentation*

203 Urine is thought to be required for inducing UPEC filamentation during development and dispersal
204 of IBCs, the extent of which was positively correlated with the specific gravity (SG) of the urine
205 samples, and was not detected in relatively dilute samples (14). Some samples with similar SG
206 also showed variation in the degree of filamentation detected, suggesting that other factors in urine
207 might be involved (14). To identify additional conditions or factors triggering UPEC filamentation,
208 we tested a potential role for urinary pH. For these experiments, we used a modified mid-scale
209 flow chamber system, similar to that described previously (14), which follows the same time-
210 course of media/urine flow described above, and allows collection of dispersed bacteria in the
211 effluent from the flow chamber and quantification of the extent of filamentation by flow cytometry.

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212   When human urine originally of pH 5.3 was adjusted to pH 4.0, the bacteria in the effluent after
213   the dispersal stage (49 h) of infection appeared as mainly rod-shaped bacteria (Fig. 6). Some of
214   the bacteria appeared smaller than typical *E. coli*—almost coccoid in shape and size. Flow
215   cytometry confirmed that the bacterial cell size, as represented by the side-scatter (SSC-A)
216   frequency distribution, in the acidified urine sample was significantly smaller than the control
217   urine; the acidified urine gave 97.5% short bacteria (defined by a threshold, as described in the
218   Methods), compared to 64.7% short for the control urine (i.e. 35.3% filamentous; Fig. 6). Many
219   bacteria within IBCs are known to be smaller coccoid-like cells compared to regular rods (9). We
220   also observed that the bacterial yield from the flow chamber was much lower in the acidified urine
221   experiments, e.g. an OD_{600} of 0.038, compared to infection with the control urine (pH 5.3) OD_{600}
222   of 0.571. These results suggested that bacteria released were killed or arrested at the IBC stage by
223   the acidified urine. The apparent poor growth or viability of bacteria from the acidified urine
224   experiment was investigated by inoculating LB with a sample of the effluent obtained from the
225   flow chamber and incubating it for 3 h at 37°C. This sample showed no increase in absorbance
226   during this time period, whereas bacteria obtained from a control infection at the same time point
227   (urine pH 5.3) more than doubled in OD_{600} during this time, from 0.571 to 1.189. This trend was
228   observed in both experimental duplicates.
229   When a sample of the pH 5.3 urine was neutralized (pH 7.0) in preparation for an infection
230   experiment, an obvious precipitate formed. This was cleared by centrifugation, and then half of
231   the supernatant was re-adjusted back to pH 5.3; both urines were tested in infections for their
232   effects on the growth and filamentation of dispersed bacteria, to determine whether components
233   in the precipitate, or the pH change, affected filamentation. Both urines caused substantial yields
234   of bacteria in the effluent (at 49 h infection), but the pH 7.0 urine gave rise to no detected
235   filamentation (Fig. 6). Similar results (high bacterial yields but no detected filamentation) were
236   observed when just EpiLife medium was maintained for the 29-49 h period (not shown).
237   Interestingly, the readjusted (pH 5.3) urine regained the capacity to induce robust filamentation of
238   the bacteria, to a similar degree as the original control urine (pH 5.3, unadjusted) (Fig. 6). Thus,
239   the pH of human urine is a key factor in the induction of UPEC filamentation.
240   ***Synthetic human urine does not induce filamentation***
241   Since a relatively high urinary SG (14) and mildly-acidic pH (above) are involved in triggering
242   filamentation, we next investigated whether these factors were sufficient to trigger filamentation

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243   with a synthetic human urine (SHU) containing a mix of pure reagents representing the common

244   solutes in human urine (22). The pH of the SHU was matched to that of a batch of human urine

245   (pH 5.6) for comparison. The SG of the real human urine and the SHU was 1.021 g/mL and 1.026

246   g/mL, respectively. Microscopy of infection effluent at 49 h post-infection showed that the human

247   urine induced bacterial filamentation, as seen previously, but the SHU supported the normal high

248   yields of bacteria in the effluent but did not induce any detectable filamentation (Fig. 6). The flow

249   cytometry data from the human urine contained a peak consistent with normal rod-shaped bacteria

250   and a large shoulder towards the right of the histogram indicating much longer bacteria (Fig. 7).

251   Some variation in the bacterial size distributions obtained from infections with batches of human

252   urine were commonly observed during this study, consistent with previous results (14). In contrast,

253   the bacterial scatter distributions from infections with SHU consistently showed only one peak of

254   small bacteria.

255   *A small molecular mass fraction of human urine can induce filamentation*

256   The above findings indicated that a high solute concentration (SG) and weak acid are necessary

257   but insufficient to cause significant filamentation during infection, suggesting that filamentation

258   also requires a urinary constituent(s) not present in SHU. To determine whether such a constituent

259   is a small or macromolecule, urine was ultra-filtered to remove all molecules of ~3000 Daltons

260   and higher. Microscopy of dispersed bacteria from infections (49 h) showed that bacteria exposed

261   to both the whole urine and the ultra-filtered urine were both dispersed with high bacterial yields

262   and were similarly filamentous, with only few short bacteria observed (Fig. 8). The fraction of the

263   urine that had not passed through the filter, containing the large molecules of 3000 Daltons and

264   higher, was diluted using sterile water back to the starting volume, so the concentration of large

265   molecules would be very similar to the whole urine, but the small molecules would be diluted.

266   This enriched high molecular mass urine was also tested in the infection model to determine

267   whether it too could induce a filamentous response in the bacteria. Microscopy of the sample of

268   (high-yield) bacteria showed they remained short and rod shaped (data not shown), indicating that

269   this portion of the urine was not able to induce filamentation, and supporting the finding that

270   components of <3000 MW were required at a concentration found in high-SG human urine for

271   triggering the filamentous response.

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272 **Discussion**

273 We have established a microfluidics-based infection model of UTI that enables real-time

274 microscopic analysis of progression of UPEC development within infected bladder epithelial cells

275 through to the IBC dispersal phase and release of rod-shaped and filamentous bacteria, thus

276 reproducing the main morphological characteristics of UPEC IBC and filament development

277 during infection. The mouse model of UTI showed similar IBCs and filamentous bacteria present

278 (9), and urine collected during human UTI also commonly contains these characteristic features of

279 infection (8). Our model is based on the mid-scale flow-chamber model developed previously (14),

280 so the results obtained will be comparable, whereas the information obtainable from each is

281 different. We have also incorporated continuous monitoring of cell viability and developed

282 improved GFP labelling of UPEC that shows robust fluorescence in a range of growth conditions

283 including the main stages of the infection.

284 Our method creates a more automated and smaller-scale infection system suited for real-time

285 microscopic visualization than previously available. Time-lapse microscopy was used to observe

286 the maturation of IBCs in mouse bladder explants (9), however to our knowledge the current study

287 is the first to demonstrate a cell-culture infection model that allows automated simultaneous

288 visualization of multiple UPEC infection experiments in real time. Using the microfluidic model,

289 we have further characterized the infection cycle, particularly in regard to the conditions required

290 for filamentous IBC dispersal at a single-cell level, and we revealed some advantages and current

291 limitations of cell culture infection models.

292 During IBC development within BECs over the first day of infection, real-time monitoring of cell

293 impermeability by SYTOX Orange exclusion demonstrated that BECs maintain their integrity and

294 surface migration while the bacteria grow internally, reflecting a likely bacterial survival

295 mechanism in which IBCs do not cause substantial changes or damage to their host until the

296 bacterial population has substantially increased in number. However, when exposed to urine for

297 day 2 of the infection, both infected and uninfected BECs became almost immediately immobile

298 (Fig. 2 and 3). Furthermore, within hours of urine exposure, individual BECs at different times

299 showed a sudden increase in autofluorescence (Movie S2), suggesting possible permeabilization

300 or other rapid change in cell metabolism that could signify a stress response (23). If

301 permeabilization occurs, the intracellular bacteria could thereby be directly exposed to the urine

302 that triggers their filamentation and dispersal. If the increased autofluorescence does not reflect

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303   permeabilization, the bacteria must sense the BEC response to the urine conditions, leading to a

304   subsequent bacterial dispersal response.

305   These results indicate that urine may not be able to support the growth or longer-term survival of

306   these BECs in culture. *In vivo*, the surface of the bladder wall is a transitional epithelium where

307   the uppermost layer of surface cells are eventually shed and replaced via differentiation of the

308   lower layer cells as they come to the surface (21, 22). The uppermost layer is made from fully

309   differentiated 'umbrella' cells, with an apical surface that deals with urine exposure in a physically

310   and chemically dynamic environment, and a basolateral surface that provides nutrition and other

311   support for the cells. The current infection model, as with previous *in vitro* models, initially has

312   the BECs growing in a monolayer of incompletely differentiated immortalized cells, likely

313   resembling intermediate cells in the bladder transitional epithelium (15, 24); these cells are likely

314   to be impacted differently by urine exposure compared to the fully differentiated surface umbrella

315   cells. A previous infection study with the PD07i cell line noted that urine was well tolerated by the

316   cultured BECs (14). By developing a real-time model, our results suggest that while the cell culture

317   models are well suited to investigate bacterial pathophysiology, they might not ideally reflect how

318   fully differentiated BECs behave late in the infection cycle *in vivo*, particularly in response to urine

319   exposure. Nevertheless, we expect that IBCs experience very similar intracellular conditions in the

320   culture models and *in vivo*, from the moment of internalization, through to establishment,

321   development, and eventually dispersal into a flow of human urine.

322   While the microfluidics-based model developed here allows efficient real-time observation, it has

323   limitations for preparative analysis of bacteria in the infection. It was found to be impractical to

324   harvest useful quantities of the dispersed bacteria, particularly long filaments, unlike the mid-scale

325   flow-chamber model where the yield of bacteria in the continuous effluent is sufficient for most

326   modern molecular and cellular analytical techniques (14). On the other hand, the microfluidic

327   infection model is more suitable for small-scale screening purposes involving direct observation

328   of infected cells, allowing up to four simultaneous infections to be run with ~100-fold less growth

329   medium. It is more cost-effective and less cumbersome to perform. One possible application for

330   the microfluidic UTI infection model could be to screen the effectiveness of newly developed

331   drugs on different stages of the infection cycle, such as IBC development. This could aid the

332   development of new treatments for UTI, as it has the advantage over non-infection-based growth

333   assays of allowing assay of UPEC growth inside BECs and during the specific morphologies we

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334   have observed during dispersal. These are important stages of the infection cycle where potential

335   treatments may be directed in future.

336   The results shown in Fig. 4 and 5 visualized IBCs growing and taking over bladder cells, causing

337   rupture and dispersal of bacteria. This has allowed an approximate timeframe to be placed on the

338   progress of the infection. At ~6-8 h after the onset of urine exposure, some infected BECs began

339   to rupture and release bacteria. However, this process initiated apparently randomly at later times

340   for other IBC's too, suggesting that a certain stage of IBC development needs to be reached before

341   dispersal of an individual IBC commences. It also underscores the low degree of infection

342   synchrony in the later stages of this complex biological system.

343   We demonstrated that extensive bacterial filamentation frequently accompanies the dispersal and

344   release from BECs (Fig. 4). Bacteria appear to encounter conditions that trigger filamentation (e.g.

345   urine exposure) while inside the BEC, or its permeable carcass, prior to the development and

346   eruption of the filamentous bacteria. This is consistent with *in vivo* data from non-real-time

347   approaches that appeared to show filamentous bacteria emerging from within a bladder cell rather

348   that forming after being released (7). Our observation that BECs may become permeabilized

349   before filaments emerge is consistent with the notion that IBCs are directly exposed to urine

350   through BEC permeabilization when they respond to these conditions by extensive bacterial

351   filamentation and dispersal (Fig. 4). However, since the dye-exclusion method cannot conclusively

352   report on permeability during urine treatment (Movie S2), it is not yet certain how urine exposure

353   may trigger the IBC response.

354   The data presented in Fig. 5 showed that some IBC's disperse from the residual BEC as a rapidly-

355   developing colony of rod-shaped bacteria, indicating that not all IBCs result in filamentous

356   bacteria and suggesting that not all IBCs experience identical conditions, or do not respond to

357   conditions in a consistent manner. The two distinct IBC developmental outcomes indicates that

358   the developmental fate of bacteria during the dispersal stage of IBC development is decided at the

359   level of whole IBC, not individual bacteria.

360   To examine the conditions that influence the switching between IBC developmental outcomes, a

361   synthetic human urine (SHU) was tested and compared to donated human urine, to determine the

362   importance of pH, relative solute concentration (specific gravity), and urinary components (small

363   and large molecules) in causing bacterial filamentation. A recently-developed formulation of SHU

364   was used (22). If this gave a similar response to human urine, we anticipated that the SHU could

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365   help improve the reproducibility of bacterial responses in the model, since different batches of
366   human urine can introduce variability in the degree of filamentation observed (14). Our
367   comparisons of SHU and human urine demonstrated that the filamentation response during IBC
368   dispersal is strongly dependent on the slightly acidic pH values of the human urine. Use of
369   neutralized or acidified human urine, or SHU of the same pH and SG as the human urine
370   (approximately pH 5.3, SG >1.02) all failed to trigger UPEC filamentation in infection (Fig. 6).
371   The previous flow-chamber study noted no obvious correlation between pH and filamentation (14),
372   however the samples were unadjusted and are expected to show only a small pH range compared
373   to the larger range of adjusted urine (pH 4 - 7) in the current study.
374   Our results suggested that either the factors in human urine that trigger filamentation are pH and
375   concentration sensitive with regards to their interactions with UPEC, or alternatively, separate
376   bacterial receptors sense pH and other urine components, and these all need to be activated in order
377   for a filamentation response to be triggered. Another study suggested that UPEC does not filament
378   in response to a simple artificial urine (25, 26), whereas the SHU used for the current research was
379   a more recently developed artificial urine (22), indicating that neither synthetic urine contains the
380   active components of human urine in triggering UPEC filamentation. Overall, our results strongly
381   support the view that filamentation is not a response to the general solute concentration or pH, but
382   it is a specific response triggered by a certain level of a component(s) in human urine and is also
383   sensitive to pH. Our investigation of human urine separated into large and small molecular weight
384   fractions by ultrafiltration clearly demonstrated that the responsible factors are present in the
385   <3000 Da fraction. The extensive human urine metabolome includes thousands of urine
386   metabolites, a large majority of which has a molecular weight less than 3000 Da (27). Identifying
387   urinary factors that trigger filamentation or other developmental steps in the UPEC infection cycle
388   would therefore benefit from metabolomic analyses or further 'top-down' fractionation of urine to
389   identify the responsible factors. Future studies on the influence and modulation of urinary pH on
390   bacterial morphology and infection progression may be possible using the murine model of UTI,
391   or larger human urine donor cohorts applying the *in vitro* model of infection described here, and
392   potential clinical trials.

14

393   **Materials and Methods**

394   *Bacterial strains and plasmid construction*

395   *E. coli* UTI89 (28), was kindly provided by J. Møller-Jensen (University of Southern Denmark).

396   Table 1 lists the oligonucleotides used in this study for plasmid construction. To construct plasmid

397   pGI5, the BamHI+EcoRI fragment from pDG57 (29) containing the pSC101 origin of replication

398   and *aadA* (for spectinomycin resistance), was ligated to a PCR product (with BamHI+EcoRI ends)

399   containing the *PlacI^Q* promoter from pNDM220 (30) to generate pGI1. The msfGFP region of

400   pDG57 was then amplified in two parts and spliced by overlap extension PCR (to silently remove

401   the internal NcoI site), followed by digestion with BglII and NcoI. This fragment was then ligated

402   to pGI1, digested with BamHI and NcoI, to create pGI2. The *PlacI^Q* promoter in pGI2 was then

403   replaced by three minimal promoters with widely varying levels of expression, *PlacI*, *PlacI^Q* and

404   *PlacI^{Q1}* (18), by annealing primers containing the promoter sequences and ligating these

405   (EcoRI+NcoI digested) to the large EcoRI+NcoI fragment of pGI2, to create the plasmids pGI3

406   (*PlacI-msfGFP*), pGI4 (*PlacI^Q-msfGFP*) and pGI5 (*PlacI^{Q1}-msfGFP*).

407   *Bladder cell culture*

408   The human bladder epithelial cell line used in this project, PD07i (kindly provided by J. Møller-

409   Jensen, University of Southern Denmark), was originally obtained from a human bladder and

410   immortalized with human papillomavirus type 16 E6E7 (31). The cells were cultured in EpiLife

411   with 60 mM calcium medium (Gibco), supplemented with 1% Human Keratinocyte Growth

412   Supplement (HKGS) (Gibco) and routinely included 1% Penicillin/Streptomicin (Gibco). The

413   cells were seeded into a T75 culture flask and left to incubate at 37 ℃ with 5 % CO2 until

414   approximately 80 % confluent, changing medium every two days. The medium was removed from

415   the flask and the cells were washed once with PBS. A solution of 0.5 % Trypsin-EDTA (Gibco)

416   was then added to cover the base of the flask and it was incubated at 37 ℃ with 5 % CO$_2$ for 10

417   minutes. The plate was agitated to aid release the cells from the surface and then a 1x volume of

418   Defined Trypsin Inhibitor (Gibco) was added and the cell suspension was transferred to a

419   centrifuge tube. The tube was centrifuged for 4 minutes at 1000 xg, the supernatant removed, and

420   the cell pellet resuspended in PBS. A volume of 100 µl was taken to obtain a cell count per mL,

421   using an M4 Multisizer Coulter cytometer (Beckmann-Coulter) equipped with a 100-micron

422   aperture tube. The cells were then centrifuged again as before, the supernatant removed, and the

423   pellet then resuspended in EpiLife medium to a concentration of 3×10^6 cells/mL. The cells were

15

424   dispensed into new T75 culture flasks to continue the culture, or into flow chambers to undergo an

425   infection with bacteria.

426   *Preparation of urine samples*

427   This study had human research ethics approval from the UTS Human Research Ethics Committee

428   (HREC REF No. 2014000452). Urine was collected soon after waking in the morning, from a

429   mildly dehydrated male donor, and stored at 4°C for 1-3 days. The samples were centrifuged at

430   4000 x g for 10 min and the supernatant was passed through a 0.2 µm membrane filter (Sartorius

431   Minisart). Specific gravity (SG) of urine samples were determined by repeat measurements of its

432   weight ratio compared to pure water, determined with a micropipette (1 mL volumes) and an

433   analytical balance. Urine with a SG of 1.020 g/mL or higher was used for infections, to promote

434   reproducible filamentation. Filter sterilized samples were stored frozen or at 4°C until required.

435   To prepare urine samples with altered pH, a sample of urine with a SG of 1.025 g/mL and pH of

436   5.33, which was known to cause filamentation, was divided into four samples. One of these was

437   used unadjusted, and a second was acidified to pH 4.0 with HCl. The third sample was neutralized

438   to pH 7.0 with NaOH, upon which a precipitate formed, which was removed by centrifugation.

439   The fourth sample of urine was similarly neutralized, the precipitate removed, and then the pH

440   readjusted to the original pH of 5.3 with HCl. To obtain the low molecular weight urine fraction,

441   filtered urine was fractionated using a Vivaspin 20 ultrafiltration spin-column with a 3000-Dalton

442   membrane filter (GE Healthcare) until approximately 75% of the original volume had passed

443   through the filter; the filtrate was used in infection studies. Synthetic Human Urine (SHU) was

444   prepared as described (22); the pH was adjusted with HCl to match the donor human urine used

445   for comparison (pH 5.0 – 5.5).

446   *Small-scale microfluidics-based infection model*

447   This microfluidic infection model was established using the CellASIC ONIX Microfluidic System,

448   with M04S-03 microfluidic plates for real-time visualization (Merck-Millipore). To prepare for an

449   infection experiment, the storage phosphate-buffered saline (PBS) from the upper parts of wells 1

450   and 8 was removed, as was the PBS from wells 6 and 7 including the bottom holes. A 10 µL

451   volume of EpiLife was pipetted into the bottom hole of Well 6 to initiate capillary flow and

452   incubated at 37°C for 30 min. The EpiLife medium in the bottom of well 6 was then replaced with

453   a 10 µl suspension of PD07i cells that had been trypsinized from an active culture and resuspended

454   at a concentration of 3×10^6 cells/ml. Well 7 was aspirated and the cell suspension was left for 10

16

455   mins to fill the culture chamber. The progress was monitored under a light microscope to check

456   qualitatively for a coverage of the chamber surface by PD07i cells. If more cells were needed,

457   another 10 µl of suspended cells was added to the bottom of Well 6, and then Well 7 was aspirated

458   again to re-initiate capillary flow. Once enough cells had filled the culture chamber, 350 µl of

459   EpiLife medium was added to Well 1, and 50 µl was added to well 7 to initiate gravity driven

460   perfusion. The plate was incubated at 37°C for 2 days to allow the bladder cells to grow and

461   approach confluency.

462   Cultures of the required bacterial strains (*e.g.* UTI89/pGI5) in LB medium were inoculated the day

463   before infection and incubated statically overnight at 37°C. The culture was centrifuged at 4000

464   rpm for 10 mins and resuspended in PBS to an optical density (OD_{600}) of 0.2 in preparation for

465   infection. Wells 6 and 7 of the flow system were aspirated including the bottom hole. A volume

466   of 10 µl of bacteria was pipetted into the bottom hole of Well 6 to initiate capillary flow and draw

467   the bacteria through the culture chamber for 20 mins. Well 6 was then aspirated and the bottom

468   hole filled with 10 µl of PBS. At this point, the Well solutions were immediately added as

469   described below, and we commenced recording the time post-infection.

470   The PBS solutions from Wells 2–4 were immediately aspirated; 110 µl of EpiLife medium was

471   added to Well 2, 210 µl of EpiLife with 100 µg/ml Gentamicin (Gm) was added to Well 3, and

472   Well 4 had 210 µl of human urine. Urine samples were obtained from a mildly dehydrated adult

473   male donor (and first micturition of the day), stored for 1-3 days at 4°C, centrifuged at 4000 xg for

474   10 min, then the supernatant was filter sterilized by passage through a 0.2 µm filter unit. Samples

475   with a density of 1.020 g/ml or greater were used for infections. For some experiments observing

476   cell permeability, 100 nM SYTOX Orange DNA stain (Thermo-Fisher) was included in the

477   medium and urine. The upper part of wells 1 and 7 were aspirated. The plate was sealed to the

478   microfluidic manifold and placed on the plate-mount stage of a Nikon Ti inverted microscope at

479   37°C to record time-lapse images of the infections. Flow pressure from the microfluidic control

480   unit was set to 1 psi for all wells. Well 2 was set to flow for 9 h and Wells 3 and 4 were each set

481   to flow for 20 h in sequence (*i.e.* until 29 h and 49 h post infection, respectively). Multiple points

482   within the chamber were selected for time-lapse microscopy. Each chamber was considered one

483   independent replicate.

484   ***Mid-scale flow-chamber infection model***

485   A modified version of the flow chamber infection model (14) was used to collect and analyze

486   dispersed UPEC. Briefly, a sterile-ported media bottle was connected with tubing via a bubble trap

487   to the flow chamber (a channel slide), through a low-pulsation peristaltic pump (Ismatec) and into

488   a waste container. To establish a PD07i monolayer, a volume of 100 µl of PD07i cells suspended

489   in EpiLife medium (3×10^6 cells/mL) was added to the flow chamber (IBIDI µ-slide I (#80176)

490   instead of the custom apparatus used previously (14)), and incubated overnight at 37 ºC with 5 %

491   CO_2. The flow system was then connected to the flow chamber, and a flow of EpiLife medium

492   without antibiotics was applied overnight (50 µL/min), and, to prepare for infection, bacterial

493   cultures (*E. coli* UTI89/pGI5) were inoculated and incubated statically at 37 °C overnight. The

494   bacterial culture was centrifuged and gently resuspended in PBS to an OD_{600} of 0.2. The flow cell

495   system was stopped and the tubing before the flow chamber was disconnected. A length of sterile

496   tubing was filled with the bacterial suspension and one end connected to the upstream port of the

497   flow chamber, ensuring no bubbles could enter the system. The other end of the tubing was

498   submerged in the bacterial suspension, avoiding bubbles, and the flow was resumed for 20 min.

499   The pump was then stopped, the short length of tubing was disconnected, the media line of tubing

500   was reconnected, and then the flow was resumed for 9 h to allow establishment of infection. The

501   pump was then stopped and all the tubing before the flow chamber was filled with EpiLife medium

502   containing gentamicin (100 µg/mL). Flow was recommenced for 20 h to kill and remove

503   extracellular bacteria and allow IBC growth and development. [To harvest the bacteria at this stage,

504   the flow chamber was disconnected and washed three times by applying 200 µL volumes of

505   Phosphate-buffered saline (PBS) to the flow chamber in-port. BEC lysis buffer (0.5 % Trypsin-

506   EDTA plus 0.1 % (v/v) Triton X-100) was then added (200 µL) to the chamber and incubated for

507   15 mins at 37 °C. The liquid was withdrawn with a pipette and then another 200 µL of lysis buffer

508   was applied. The two samples were pooled, centrifuged at 5000 x g for 5 min and then bacteria

509   were fixed by resuspension in 2% (w/v) formaldehyde in PBS and stored at 4 °C.] To continue to

510   the dispersal stage after the 20 h flow of EpiLife+Gm, the tubing before the flow chamber was

511   disconnected and filled with prepared urine as described above, then connected to the urine bottle

512   and flow chamber, and the flow continued for another 20 h. To harvest the bacteria, the flow

513   chamber was disconnected at both ends, and the liquid supernatant inside the chamber withdrawn

514   and retained. PBS (200 µl) was then added to the chamber, pipetted back and forth, and then

515   withdrawn and pooled with the supernatant. This sample was centrifuged at 5000 x g for 5 min

516   and the pellet was gently resuspended in 2% formaldehyde and stored at 4 °C.

517   *Microscopy*

518   Samples of fixed bacteria (2 µL) were mounted on a ~170 micron thick 1% agarose pad in PBS

519   prepared on a slide. The bacteria were viewed using a Nikon Ti epifluorescence microscope using

520   a Plan Apo λ 1.4 NA phase-contrast oil objective. For fluorescence microscopy, Nikon filter-sets

521   for GFP or TRITC/Texas Red (for SYTOX Orange detection) were used. Images were recorded

522   using a Nikon DS-Qi2 camera and visualized and analyzed using Fiji (32).

523   *Flow cytometry*

524   Bacterial samples were analyzed with a BD LSRII flow cytometer to record forward scatter (FSC),

525   side scatter (SSC, trigger) and fluorescence (GFP) signal for 100,000 events per sample, with the

526   side scatter also set as the threshold. To further analyze and display the flow cytometry data,

527   FlowJo (Ver 10) was used. A histogram was generated, in which the frequency data were

528   normalized to the mode of each sample, and then data were gated to distinguish between the short

529   and filamentous bacteria, by defining a "short population" based on a control (UTI89/pGI5 grown

530   to mid-log phase in LB), which defined a gate (arbitrary cutoff) to contain 99% of events; this was

531   then applied to each sample equally, to define the fraction of cells showing smaller ('short cells')

532   or larger ('filamentous') values of side-scatter (area) parameter (SSC-A) than the cutoff. The

533   displayed frequency distributions are consistent and representative of at least two independent

534   replicate experiments.

Acknowledgements

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References

540 **References**

541 1.   Phan MD, Peters KM, Sarkar S, Lukowski SW, Allsopp LP, Gomes Moriel D, Achard ME, Totsika M,
542   Marshall VM, Upton M, Beatson SA, Schembri MA. 2013. The serum resistome of a globally
543   disseminated multidrug resistant uropathogenic Escherichia coli clone. PLoS Genet 9:e1003834.
544 2.   Connell I, Agace W, Klemm P, Schembri M, Marild S, Svanborg C. 1996. Type 1 fimbrial expression
545   enhances Escherichia coli virulence for the urinary tract. Proc Natl Acad Sci U S A 93:9827-32.
546 3.   Mulvey MA. 2002. Adhesion and entry of uropathogenic Escherichia coli. Cell Microbiol 4:257-71.
547 4.   Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. 2003. Intracellular bacterial
548   biofilm-like pods in urinary tract infections. Science 301:105-7.
549 5.   Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ. 2000. Type 1 pilus-mediated bacterial
550   invasion of bladder epithelial cells. The EMBO journal 19:2803-12.
551 6.   Mulvey MA. 1998. Induction and Evasion of Host Defenses by Type 1-Piliated Uropathogenic
552   Escherichia coli. Science 282:1494-1497.
553 7.   Mulvey MA, Schilling JD, Hultgren SJ. 2001. Establishment of a persistent Escherichia coli reservoir
554   during the acute phase of a bladder infection. Infection and immunity 69:4572-9.
555 8.   Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ. 2007. Detection of intracellular
556   bacterial communities in human urinary tract infection. PLoS medicine 4:e329.
557 9.   Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ. 2004.
558   Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract
559   pathogenesis. Proceedings of the National Academy of Sciences of the United States of America
560   101:1333-8.
561 10. Justice SS, Hunstad DA, Cegelski L, Hultgren SJ. 2008. Morphological plasticity as a bacterial
562   survival strategy. Nature reviews Microbiology 6:162-8.
563 11. Justice SS, Hunstad DA, Seed PC, Hultgren SJ. 2006. Filamentation by Escherichia coli subverts
564   innate defenses during urinary tract infection. Proceedings of the National Academy of Sciences
565   of the United States of America 103:19884-9.
566 12. Horvath DJ, Jr., Li B, Casper T, Partida-Sanchez S, Hunstad DA, Hultgren SJ, Justice SS. 2011.
567   Morphological plasticity promotes resistance to phagocyte killing of uropathogenic Escherichia
568   coli. Microbes and infection / Institut Pasteur 13:426-37.
569 13. Moller J, Emge P, Vizcarra IA, Kollmannsberger P, Vogel V. 2013. Bacterial filamentation
570   accelerates colonization of adhesive spots embedded in biopassive surfaces. New Journal of
571   Physics 15:125016.
572 14. Andersen TE, Khandige S, Madelung M, Brewer J, Kolmos HJ, Moller-Jensen J. 2012. Escherichia
573   coli uropathogenesis in vitro: invasion, cellular escape, and secondary infection analyzed in a
574   human bladder cell infection model. Infection and immunity 80:1858-67.
575 15. Berry RE, Klumpp DJ, Schaeffer AJ. 2009. Urothelial cultures support intracellular bacterial
576   community formation by uropathogenic Escherichia coli. Infection and immunity 77:2762-72.
577 16. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli
578   K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States
579   of America 97:6640-5.
580 17. Urban JH, Vogel J. 2007. Translational control and target recognition by Escherichia coli small
581   RNAs in vivo. Nucleic Acids Res 35:1018-37.
582 18. Glascock CB, Weickert MJ. 1998. Using chromosomal lacIQ1 to control expression of genes on
583   high-copy-number plasmids in Escherichia coli. Gene 223:221-31.
584 19. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. 2006. Engineering and
585   characterization of a superfolder green fluorescent protein. Nat Biotechnol 24:79-88.

586   20.   Ito K, Minamiura N, Yamamoto T. 1984. Human urine DNase I: immunological identity with human
587   pancreatic DNase I, and enzymic and proteochemical properties of the enzyme. J Biochem
588   95:1399-406.
589   21.   Yasuda T, Nadano D, Awazu S, Kishi K. 1992. Human urine deoxyribonuclease II (DNase II)
590   isoenzymes: a novel immunoaffinity purification, biochemical multiplicity, genetic heterogeneity
591   and broad distribution among tissues and body fluids. Biochim Biophys Acta 1119:185-93.
592   22.   Ipe DS, Ulett GC. 2016. Evaluation of the in vitro growth of urinary tract infection-causing gram-
593   negative and gram-positive bacteria in a proposed synthetic human urine (SHU) medium. J
594   Microbiol Methods 127:164-71.
595   23.   Surre J, Saint-Ruf C, Collin V, Orenga S, Ramjeet M, Matic I. 2018. Strong increase in the
596   autofluorescence of cells signals struggle for survival. Sci Rep 8:12088.
597   24.   Apodaca G. 2004. The uroepithelium: not just a passive barrier. Traffic 5:117-28.
598   25.   Brooks T, Keevil CW. 1997. A simple artificial urine for the growth of urinary pathogens. Lett Appl
599   Microbiol 24:203-6.
600   26.   Klein K, Palarasah Y, Kolmos HJ, Moller-Jensen J, Andersen TE. 2015. Quantification of
601   filamentation by uropathogenic Escherichia coli during experimental bladder cell infection by
602   using semi-automated image analysis. J Microbiol Methods 109:110-6.
603   27.   Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C, Bjorndahl TC, Krishnamurthy R, Saleem
604   F, Liu P, Dame ZT, Poelzer J, Huynh J, Yallou FS, Psychogios N, Dong E, Bogumil R, Roehring C,
605   Wishart DS. 2013. The human urine metabolome. PLoS One 8:e73076.
606   28.   Chen SL, Hung CS, Xu J, Reigstad CS, Magrini V, Sabo A, Blasiar D, Bieri T, Meyer RR, Ozersky P,
607   Armstrong JR, Fulton RS, Latreille JP, Spieth J, Hooton TM, Mardis ER, Hultgren SJ, Gordon JI. 2006.
608   Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a
609   comparative genomics approach. Proceedings of the National Academy of Sciences of the United
610   States of America 103:5977-82.
611   29.   Ghosal D, Trambaiolo D, Amos LA, Lowe J. 2014. MinCD cell division proteins form alternating
612   copolymeric cytomotive filaments. Nat Commun 5:5341.
613   30.   Gotfredsen M, Gerdes K. 1998. The Escherichia coli relBE genes belong to a new toxin-antitoxin
614   gene family. Mol Microbiol 29:1065-76.
615   31.   Klumpp DJ, Weiser AC, Sengupta S, Forrestal SG, Batler RA, Schaeffer AJ. 2001. Uropathogenic
616   Escherichia coli potentiates type 1 pilus-induced apoptosis by suppressing NF-kappaB. Infect
617   Immun 69:6689-95.
618   32.   Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C,
619   Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012.
620   Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-82.

621

622

623 **Figure Legends**

624 **Figure 1. Microfluidic model for the intracellular UPEC and release stages of UTI.**

625 Diagrammatic representation of the CellASIC Onix microfluidics plate showing the contents of

626 each well that were sequentially pumped through the main chamber during the defined stages of

627 the infection. The lower panels show phase-contrast and fluorescence microscopy images of the

628 infection chamber surface at the end of the intracellular growth stage (29 h post-infection),

629 showing a well-developed IBC, and at the end of the dispersal phase (49 h post-infection), showing

630 filamentous UTI89/pGI5 in amongst BECs. Scale bars are 5 μm.

631 **Figure 2. PD07i bladder cells infected with UTI89/pGI5 are mobile.** Time-lapse microscopy

632 showing 3 h intervals during the 9-29 h stage of infection, with a flow of EpiLife + Gentamicin

633 (Gm). A phase-contrast and GFP channels overlay (left) with SYTOX Orange channel (right) are

634 shown for the indicated timepoints after switching to Gm-containing medium. Infected bladder

635 cells (black, white and blue arrowheads, which track individual BECs) appeared at different

636 positions at each time point. SYTOX Orange staining indicated that most infected and uninfected

637 bladder cells were not permeable, whereas permeable, assigned-dead bladder cells are indicated

638 by red arrowheads; these occasionally drifted detached from the surface during the movie. Scale

639 bars are 20 μm (60X oil objective). Refer to Movie S1 for the full set of time-lapse images.

640 **Figure 3. Bladder cells become immobile after exposure to urine.** Microscopy images taken 20

641 min before urine exposure, immediately after switching to urine (0 h), and 10 h into the urine

642 exposure. Dead bladder cells have detached from the monolayer and taken up SYTOX Orange

643 stain (e.g. white arrowheads), but the staining disappears soon after urine exposure (within 10-20

644 min). Scale bars are 20 μm (40X objective); all GFP exposures = 50 ms, all SYTOX exposures =

645 100 ms. Refer to Movie S2 for the full set of time-lapse images.

646 **Figure 4. Filamentous bacteria emerging from an infected bladder cell.** Phase (A) and GFP

647 fluorescence (B) time-lapse microscopy, showing an IBC developing from within an infected

648 bladder cell during the 20 h urine exposure. The bacteria overwhelmed the bladder cell causing it

649 to rupture and release filamentous bacteria. Scale bars are 10 μm, 40X objective, all GFP exposures

650 = 50 ms. Refer to Movie S3 for the full set of time-lapse images.

651 **Figure 5. Short bacteria emerging from an infected bladder cell.** Phase (A) and GFP

652 fluorescence (B) time-lapse microscopy showing an IBC developing from within an infected

22

653 bladder cell during the 20 h urine exposure. The bacteria overwhelmed the bladder cell causing it

654 to rupture and release many short bacteria, and no detected filaments. Scale bars are 10 µm, 40X

655 objective, all GFP exposures = 50 ms. Refer to Movie S4 for the full set of time-lapse images.

656 **Figure 6. Urine pH controls UPEC filamentation.** Phase-contrast microscopy and flow

657 cytometry of UTI89/pGI5, harvested from the flow chamber infection model after day 2, with

658 exposure to urine under different pH conditions; control (normal human urine, red, top left),

659 acidified urine (blue, top right), neutralized urine (amber, bottom left), and pH-readjusted urine

660 (green, bottom right). Scale bars are 5 µm. The lower panel shows flow cytometry frequency

661 distributions, normalized to the sample mode, indicating the fraction of cells below or above a

662 side-scatter area (SSC-A) cutoff value, defined as the SSC-A below which there is 99% of cells of

663 a UTI89/pGI5 LB mid-log culture. Sample colors correspond to the image frames above.

664 **Figure 7. Synthetic human urine does not induce UPEC filamentation.** Phase-contrast

665 microscopy and flow cytometry of UTI89/pGI5, harvested from the flow chamber infection model

666 after day 2, with exposure to human urine (red) or synthetic human urine (SHU) (blue). Scale bars

667 are 5 µm. Flow cytometry of infection effluent with human urine showed a left hand peak with a

668 large right hand shoulder indicating a mixed population of short and filamentous bacteria (12.2%

669 filamentous, according to the cut-off); the bacteria exposed to SHU were represented by a narrow

670 left hand peak indicating a population of short bacteria of a similar length (99% short).

671 **Figure 8. The urine small-molecule fraction supports robust UPEC filamentation.** Phase-

672 contrast microscopy and flow cytometry of UTI89/pGI5, harvested from the flow chamber

673 infection model after day 2, with exposure to whole human urine (red) or small molecule human

674 urine (<3000 Da fraction, blue). Scale bars are 5µm. Flow cytometry showed very similar curves

675 for both urine types, a left-hand peak with a large right hand shoulder indicating a mixed

676 population of short and filamentous bacteria, 12.2% of bacteria for whole urine and 12.7% of small

677 molecule urine were filamentous.

23

678   **Table 1.** Oligonucleotides used in this study.*

Name	Sequence (5' to 3')	Description
PlacIq_F	GGGCCCGAATTC**GCGGATTTGAACGTTGCG**	Amplify the *PlacI*^{Q}
PlacIq_R	GGGCCCGGATCCATTAATTCCCATGG**TCACCA CCCTGAATTGACTC**	promoter from the plasmid pNDM220
msfGFP_F	GGGCCCCCATGG**GTAAAGGTGAAGAACTGTTC ACC**	Amplify first half of msfGFP from
msfGFP_noNco_R	**CCAGAGTCGGCCACGGAACCGGCAGTTTAC**	pDG57
msfGFP_R	GGGCCCAGATCTTTAGGATCC**TTTGTAGAGTT CATCCATGCC**	Amplify second half of msfGFP from
msfGFP_noNco_F	**GTAAACTGCCGGTTCCGTGGCCGACTCTGG**	pDG57
PlacI_F	AATTC**GCGCAAAACCTTTCGCGGTATGGCATG ATAGCGCCCGGTCTAGAGGAGGTACTA**C	Annealed to create
PlacI_R	CATGG**TAGTACCTCCTCTAGACCGGGCGCTAT CATGCCATACCGCGAAAGGTTTTGCGC**G	the promoter *PlacI*
PlacIQ_F	AATTC**GTGCAAAACCTTTCGCGGTATGGCATG ATAGCGCCCGGTCTAGAGGAGGTACTA**C	Annealed to create
PlacIQ_R	CATGG**TAGTACCTCCTCTAGACCGGGCGCTAT CATGCCATACCGCGAAAGGTTTTGCAC**G	the promoter *PlacI*^{Q}
PlacIQ1_F	AATTC**TTGACACCACCTTTCGCGGTATGGCAT GATAGCGCCCGGTCTAGAGGAGGTACTA**C	Annealed to create
PlacIQ1_R	CATGG**TAGTACCTCCTCTAGACCGGGCGCTAT CATGCCATACCGCGAAAGGTGGTGTCAA**G	the promoter *PlacI*^{Q1}

679   * Relevant restriction sites are underlined. Complementary regions in bold.

680

24

Pre-Infection | Post-Infection reservoirs

EpiLife | EpiLife | EpiLife + Gm | Urine | Spare | Inlet for cells | Infection Chamber | Waste

B1 | B2 | B3 | B4 | B5 | B6 | | B7 | B8

29 h Post-Infection (after Epilife + Gm)

Phase | GFP | 5 µm

49 h Post-Infection (after urine)

Phase | GFP | 5 µm

Phase + GFP	SYTOX Orange	Phase + GFP	SYTOX Orange

20 min pre-urine exposure | Start of urine exposure | 10 h urine exposure

Phase-contrast

SYTOX Orange channel

GFP channel

20 µm

A   Filamentous dispersal - Phase

B   Filamentous dispersal - GFP

A   Rod dispersal - Phase   B   Rod dispersal - GFP

Human Urine

Synthetic Human Urine (SHU)

5 μm

Whole human urine

Small molecule human urine

5 μm