Evasion of Toll-like Receptor Recognition by *Escherichia coli* is mediated via Population Level Regulation of Flagellin Production

Short Title: TLR5 Evasion by Uropathogenic *E. coli*

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27 **Abstract (300)**

28 Uropathogenic *Escherichia coli* (UPEC) is a major cause of urinary tract infections. Analysis
 29 of the innate immune response in immortalised urothelial cells suggests that the bacterial
 30 flagellar subunit, flagellin, is key in inducing host defences. A panel of 40 clinical uro-
 31 associated *Escherichia coli* isolates recovered from either asymptomatic bacteruria (ASB),
 32 cystitis or pyelonephritis patients, were characterised for motility and their ability to induce
 33 an innate response in urothelial cells stably transfected with a NFκB luciferase reporter.
 34 Twenty-four isolates (60%) were identified as motile with strains recovered from cystitis
 35 patients exhibiting a bipolar motility distribution pattern ($P < 0.005$) and associated with a 2-
 36 5 fold increase in NFκB signalling. Although two isolates were associated with swarm sizes
 37 of >7 cm and NFκB activities of >30 fold ($P = 0.029$), data overall suggested bacterial motility
 38 and the NFκB signalling response were not directly correlated. To explore whether the
 39 signalling response reflected antigenic variation flagellin was purified from 11 different
 40 isolates and the urothelial cell challenges repeated. Purified flagellin filaments generated
 41 comparable (30.4 ± 1.8 to 46.1 ± 2.5 fold, $P = \text{NS}$) NFκB signalling responses, irrespective of
 42 either the source of the isolate or H-serotype. These data argued against any variability
 43 between isolates being related to flagellin itself. To determine the roles, if any, of flagellar
 44 abundance in inducing these responses flagellar hook numbers of a range of cystitis and
 45 ABU isolates were quantified using a plasmid encoded flagellar hook gene *flgEA240C*. Foci
 46 data suggested isolates were averaging between 1 and 2 flagella per cell, while only 10 to
 47 60% each isolates population exhibited foci. These data suggested selective pressures exist
 48 in the urinary tract that allow uro-associated *E. coli* strains to maintain motility exploiting
 49 population heterogeneity to prevent host TLR5 recognition.

50

51 Introduction

52

53 Urinary tract infections (UTIs) are among the most common bacterial infections suffered by
 54 individuals of all ages. They affect an estimated 150 million people worldwide including
 55 children, young adults and older populations (1). Infections are often painful and debilitating,
 56 associated with a wide range of pathogens, but the majority (70-80%) link to the bacterial
 57 uropathogen *Escherichia coli* (2). Regardless of the uropathogen, treatment options remain
 58 limited with antibiotics being the first choice therapeutic. Treatment consequences, namely
 59 multi-drug resistant bacteria, often underpin persistent or rUTIs and has driven the urologic
 60 community to work collaboratively to adopt antibiotic stewardship programmes (3)

61

62 Research to date suggests UTIs link to genotypic and phenotypic variation in both the host
 63 and the uropathogen (4,5). At present it is assumed that the relationship between an
 64 individual's susceptibility and bacterial virulence determines the balance between tolerance
 65 of invading pathogens and the mounting of an immune response, which in turn dictates the
 66 course of infection and subsequent recurrence (6-8). *Escherichia coli* reside naturally in the
 67 gastrointestinal tract, but are able to migrate from the anus, colonise the vaginal and
 68 periurethral areas, then ascend to the bladder causing asymptomatic infection (ABU) or
 69 acute cystitis (5). However, our understanding of the associated host-microbe interactions
 70 is compounded by the observation that the same or related strains can lead to both
 71 symptomatic UTI and ABU. One outcome is that ABU patients, particularly the elderly, are
 72 often given antibiotics without justification due to clinical uncertainty (1).

73

74 While UPEC harbour a large array of virulence determinants, the ability to cause disease is
 75 dependent on the ability of the bacterium to ascend the urinary tract through adherence
 76 (fimbriae driven) and flagella-based motility (2). Moreover *in vivo* studies using genetically

77 engineered UPEC strains and mice UTI models support flagella as being a key factor in the
 78 aetiology of an UTI (9-11). The bacterial flagellum is a macromolecular, self-assembling
 79 nano-machine whose genetics, assembly process and mechanisms of action during host-
 80 microbe interactions are well-documented (12-17). *E. coli* is known to produce 2-8 flagella
 81 per cell arranged peritrichously across the cell surface and, is characterised genetically, by
 82 approximately 60 flagellar genes organised into three loci: *flg*, *flh* and *fli* that function to
 83 orchestrate flagellar assembly and rotation (18). Evidence supports flagellar assembly and
 84 function to be coupled to flagellar gene expression by a complex transcriptional hierarchy
 85 (19). Additionally, tight control of flagellar gene expression enables *E. coli* to efficiently pass-
 86 through ON/OFF phases of motility that can be exploited and used advantageously during
 87 host-microbe interactions (20).

88

89 In humans, uropathogens such as UPEC are sensed via TLR5 receptors, which detect
 90 flagellin: the major subunit of the flagellum filament (21). TLR5 activation results in the rapid
 91 release of urothelial host defence agents including cytokines and defensins that function
 92 individually or collectively to kill potential uropathogens (22,23). However, using urine and
 93 employing *in vitro* chemotaxis assays Herrmann and Burman (1985) reported that only 68%
 94 (19/28) of *E. coli* isolates associated with cystitis, or an UTI, were motile (24). Yet, there is
 95 strong evidence to support uropathogenic *E. coli* (UPEC) exploiting flagellar-mediated
 96 movement to establish the initial ascending colonisation of the bladder from the urethra
 97 (11,25). Lane et al (2005) and Wright et al (2005) both concluded that motility provided
 98 UPEC a competitive advantage over non-motile UPEC strains in establishing an UTI in
 99 murine models. A key challenge therefore is to understand what triggers potential
 100 uropathogenic bacteria to regulate their motility, ascend the urethra and infect the bladder.

101

102 Using clinically derived uro-associated *E. coli* isolates data are presented suggesting a
103 regulatory mechanism linked to population heterogeneity that maintains motility within a
104 bacterial population, but at levels below a threshold required for innate immune recognition.
105

Results

Uro-associated E. coli motility and the urothelial innate response.

Forty uro-associated *E. coli* isolates were curated from patients presenting with either cystitis, pyelonephritis, asymptomatic bacteriuria (ABU) or UTI-associated bacteraemia. Semi-quantitative agar assays measuring the size of a bacterial swarm after 8 hrs (Figure 1A) were used to assess motility of these isolates and 24 (60%) were identified as motile (Fig 1A & B, Table S1). In general, the swarms of isolates recovered from ABU patients measured between 0.8 and 5.4 cm, while cystitis strains exhibited a bipolar motility distribution pattern with strains swarming less (n=6) or greater (n=4) than 5.4 cm respectively (Fig 1B; $P < 0.005$).

The impact of bacterial motility on the urothelial innate response was assessed *in vitro* using heat-killed isolates (1×10^5 CFU/ml) and bladder RT4 cells stably transfected with a NF κ B luciferase reporter (26). Following these challenges 33 (83%) of all the isolates were associated with a 2-5 fold increase in NF κ B signalling (Fig 1B & C), with 7 of the motile isolates associated with increases of >5-fold. Two isolates recovered from cystitis patients were associated with NF κ B activities of >30 fold (Fig 1C; $P = 0.029$) and swarm sizes of >7 cm (Fig 1B). These data suggested that bacterial motility linked to a NF κ B signalling response, but that the two were not directly correlated.

Urothelial responses to flagellins prepared from uro-associated E. coli isolates

UTIs are ascending infections and urothelial cells respond to potential uropathogens via flagellin detection, TLR5 signalling and the release of antimicrobial killing and pro-

132 inflammatory agents (26). TLR5 proteins recognise a conserved motif found in the majority
 133 of flagellins (27), which in *E. coli* is referred to as the H-antigen with to date 53 flagellin or
 134 H-serotypes being identified (28) (Table S1). To explore whether the signalling response
 135 reflected antigenic variation flagellin was purified from 11 different uro-associated *E. coli*
 136 isolates (Table S1: Lab IDs PYL3398-PYL3424) and the urothelial cell challenges repeated
 137 (Fig 2A). Purified flagellin filaments generated robust and comparable (30.4 ± 1.8 to 46.1 ± 2.5
 138 fold, $P = \text{NS}$) NF κ B signalling responses, irrespective of either the source of the isolate or
 139 H-serotype. These data argued against any variability between isolates being related to
 140 flagellin itself.

141
 142 TLR-signalling activates a complex signalling cascade that leads to the degradation of I κ B α
 143 and NF κ B release that activates gene expression (29). Smith et al (2003) using transfected
 144 Chinese hamster ovary cells, showed TLR5 recognition of flagellin to be dose-dependent
 145 (30). A dose-dependent response to flagellin, measured through I κ B α protein levels and
 146 NF κ B induction was also observed in RT4 urothelial cells (Fig 2B and C). Interestingly, using
 147 purified flagellin from *E. coli* UTI3408 experiments suggested a peak response at
 148 approximately 2.5 ng/ml.

149
 150 *Urothelial responses to outer membranes prepared from uro-associated E. coli isolates*

151
 152 Data from *in vitro* and *in vivo* studies, both murine and clinical, suggest TLR4 signalling as
 153 well as TLR5 impacts UTI host-microbe interactions (21,31-33). TLR4 recognises
 154 lipopolysaccharide, the major outer membrane (OM) component of Gram-negative bacterial
 155 species such as UPEC (34). Therefore, the potential roles of TLR4 and LPS were explored
 156 to help explain the differing NF κ B signalling responses observed during whole bacterial cell
 157 challenges. Outer membrane preparations of four clinical isolates (3 motile [PYL3398,

158 UTI3408 and UTI3412] (Table S1) and 1 non-motile [ABU3416]) and two control strains
 159 NCTC10418 and CFT073, were used to challenge RT4 (TLR⁺) cells and RT4 cells where
 160 either TLR4 or TLR5 expression had been inhibited by siRNA knockdown (Fig 2D). The
 161 innate response was determined through measurement of the pro-inflammatory cytokine IL-
 162 8. Data presented in Fig 2D showed that the IL-8 responses to flagellin (50 ng/ml) and whole
 163 bacterial cell challenges were significantly reduced in the TLR5^{siRNA} cells (P = 0.0013). While
 164 a two-fold reduction in IL8 was observed in the NCTC14028 challenged TLR4^{siRNA} cells.
 165 This decrease was not significant and not mirrored in either the flagellin or CFT073
 166 challenges. Challenges with OM sample preparations supported reduced IL-8
 167 concentrations overall, but again a significant reduction was detected only in the TLR5
 168 silenced cells (Fig 2D P < 0.001). While these data suggested the OM preparations maybe
 169 contaminated with flagellin they also supported minor roles for LPS and TLR4 in the RT4
 170 bladder cell innate response to an acute infection.

171

172 *Correlating urothelial responses to flagellar abundance amongst uro-associated E. coli* 173 *isolates*

174

175 Data in Fig 1 and 2 indicated that flagellin isolated from a range of uro-associated *E. coli*
 176 induced an innate response as shown by NFκB signalling and effector (IL-8) responses. To
 177 determine the roles, if any, of flagellar abundance in inducing these responses flagellar hook
 178 numbers of cystitis (5), ABU (10) and PYL (1) isolates were quantified (Fig 3A) using the
 179 plasmid encoded flagellar hook gene *flgEA240C* (20). The control *E. coli* strain NCTC10418
 180 averaged 2.5 foci per cell, while foci data suggested the clinical isolates and CFT073 were
 181 averaging between 1 and 2 flagella per cell (P = 0.148) (Fig 3B, x-axis).

182

183 However, only 10 to 60% of the CFT073 and clinical isolate cell populations exhibited
 184 FlgEA240C foci compared to 80% of the NCTC10418 cell population (Fig 3B, y-axis).
 185 Moreover, using NCTC10418 as the control and exploiting IL-8 concentrations resulting from
 186 RT4 urothelial cells challenge experiments, identified a link between flagellar numbers and
 187 the host response (Fig 3C $P = 0.013$). These data suggested selective pressures exist in
 188 the urinary tract that allow uro-associated *E. coli* strains to maintain motility but exploit
 189 population heterogeneity to prevent host TLR5 recognition and bacterial killing.

190

191 The flagellar system is regulated at the expression level by a transcriptional hierarchy
 192 controlled by the master transcriptional regulatory FlhD₄C₂ (35). FlhD₄C₂ levels are sensitive
 193 to a wide range of regulatory mechanisms that include transcription, translation and protein
 194 stability (36-39). Population data (Fig 3B) suggested that the number of flagella observed
 195 linked to reduced flagellar gene expression. To examine this further a high copy number
 196 plasmid encoding *flhDC* was transformed into the control strain NCTC10418 and UTI3408
 197 (25% Fla⁺; 1.66 Fla/cell; 0.19 relative IL-8 production: Table S1). RT4 urothelial cell
 198 challenge experiments performed using these transformed strains showed that increasing
 199 *flhDC* expression supported a two-fold increase in IL-8 concentrations for UTI3408
 200 compared to no change for NCTC10418 (Fig 3D).

201

202 Discussion

203

204 Motility is a well-recognised pathogenicity, virulence and/or colonisation factor for a wide
 205 range of bacterial species including uropathogenic *E. coli* (UPEC) (40). However, motility
 206 links to flagellin production, which is the bacterial ligand for the mammalian host receptor
 207 TLR5. TLR5 activation releases host bacterial killing agents including cytokines and
 208 antimicrobial agents that facilitate bacterial killing, and clearance from potential colonisation
 209 and/or infection sites (21,26,30). Data from this study exploiting clinical isolates associated
 210 with UTIs suggest that uro-associated *E. coli* exploit population heterogeneity to maintain
 211 motility, but prevent the TLR5-dependent activation of the host innate immune response
 212 (41). Essentially these bacterial populations manipulate their flagellar production so they can
 213 survive and/or colonise the urinary tract, but remain under the host radar. It has been
 214 reported that *E. coli* swims efficiently with only one flagellum per cell (42) and using a
 215 FlgEA240C foci labelling approach uro-associated clinical isolates and the UPEC model
 216 strain CFT073 cultured *in vitro* were characterised by 1 to 2 flagella per cell (Fig 3). Together
 217 these data support *E. coli* motility and survivability within the lower urinary tract environment.
 218 However, once motility is enhanced through increased flagellar production, modelled *in vitro*
 219 using strain 3408 (Fig 3D), these microbes become visible to urothelial cell TLRs. Detection
 220 is associated with the release of a plethora of host defence molecules, shown here in these
 221 studies by elevated IL-8 concentrations (Fig 3D).

222

223 Flagellar systems have been shown to be subject to multiple regulatory controls (43). For
 224 example, *Salmonella enterica* generates a bipolar Fla⁻/Fla⁺ population in response to either
 225 nutritional and/or cell envelope stresses (44) while *Caulobacter crescentus* divides
 226 asymmetrically to produce one motile cell each division, ensuring a subpopulation of motile
 227 progeny within a growing population (45). While urine is a medium that sustains microbial

228 growth, it is well recognised to be nutritionally weak when compared to normal laboratory
 229 growth conditions (46). Therefore, it could be argued that uro-associated *E. coli* completely
 230 switching off flagellin synthesis to evade TLR5 recognition and the host immune response
 231 is not compatible with its survival. However, exploiting population heterogeneity to regulate
 232 environmental flagellin concentrations ensures microbial survivability and potentially host
 233 colonisation. Additionally, the concept that immune evasion i.e. host TLR5 recognition of
 234 flagellin proteins drives uro-associated *E. coli* to downregulate flagellar production may help
 235 unravel the pathogenesis of asymptomatic bacteria, defined as the presence of bacteria in
 236 the urinary tract without inflammatory symptoms.

237

238 It was interesting, however, that following *in vitro* culture the motility and NF κ B observations
 239 (Fig 2B) did not differentiate between the ASB and cystitis strains. During active UTIs in
 240 humans it has been reported that infecting bacteria need to divide rapidly to survive the host
 241 innate response. Doubling times have been estimated to be between 17 to 34 minutes and
 242 averaging 22 minutes (47). This rapid growth response characterised by upregulation of
 243 UPEC translational machinery results in high cell densities that are orchestrated to both
 244 overwhelm and escape the host defences (48). The latter is supported by growth
 245 experiments using steady state chemostat cultures where faster growing *E. coli* produce
 246 more flagella (Sim 2017).

247

248 However, a key question relates to the cues in the urogenital tract that trigger increased
 249 growth and UPEC infections. It is generally accepted that low nutrient conditions up-regulate
 250 *E. coli* flagellar synthesis via activation of the *flhDC* operon (49) although other signals
 251 including urine osmolality and pH cannot be ignored (50). Studies in *Salmonella* grown in
 252 low nutrient conditions have shown that non flagellar regulators such as RfIP, a regulator
 253 that modulates ClpXP recognition of FlhD₄C₂, can impact the master regulator FlhD₄C₂

254 activity and hence flagellin synthesis (39,44,49,51). Whether comparable regulators function
 255 to trigger flagellar growth in uro-associated *E.coli* is not known although NarL, ModE, MetJ,
 256 GadE and YdeO, all sensors of environmental cues, have been identified as playing
 257 potential roles in infection-specific UPEC gene expression (48).

258

259 Population heterogeneity is not an original concept and has been shown to be exploited by
 260 a number of bacterial species to retain a selective advantage particularly during growth in
 261 specific environmental niches (41,52,53). However, its exploitation by uro-associated *E. coli*
 262 to regulate flagellin synthesis and avoid the host defences is novel. This study was not
 263 designed to identify the regulatory mechanisms functioning to control flagellin synthesis in
 264 the urogenital tract, but environmental and genetic cues including population densities, urine
 265 osmolality and electrolytes, urinary and bacterial metabolites, and pH need to be
 266 investigated further.

267

268 **Materials and Methods**

269

270 ***Strains and General Microbiology***

271

272 *E. coli* strains used in the study have either been previously described (26). Strains PYL3398
273 to ABU3710 were a kind donation from the Diagnostic Microbiology Unit at the Freeman
274 hospital, Newcastle NHS Trust, Newcastle upon Tyne between 2010 and 2012 (Table S1
275 and S2). No ethics were necessary for the use of these strains as the researchers did not
276 have access to clinical records and the only information provided by the unit was the type
277 of UTI associated with each isolate. Strains ABU4738-ABU4745 came from the clinical study
278 of Drage et al (2019) that was conducted under ethically approved study protocols (ref: REC-
279 14-NE-0026).

280

281 Strains used during this study were propagated in or on Luria-Bertani (LB) medium using
282 1.5% agar for plates. Incubation, unless stated otherwise, was overnight at 37°C with liquid
283 cultures aerated by orbital shaking at 160 rpm. All motility assays were performed by either
284 direct inoculation using a toothpick or inoculating 3 µl of an overnight culture onto motility
285 agar (1% Tryptone, 0.5% NaCl, 0.3% Agar) and incubating for 8 hours at 30°C. Images of
286 motility swarms were digitally captured, and the vertical and horizontal diameter measured
287 to generate an average swarm distance using ImageJ. All swarm assays were performed
288 with a minimum of three independent colonies. Transformation of the plasmids *pflhDC* or
289 *pBADflgEA240C* were performed by electroporation as described previously (20). Selection
290 for plasmids was performed using either 100 µg/ml Ampicillin or 50 µg/ml Kanamycin.
291 *pflhDC* was generated by cloning a PCR product using the primers *flhD*-42FBam
292 [ggcggatccGGGTGCGGCTACGTCGCAC] and *flhC*+616RBam

293 [ggcggatccCGCTGCTGGAGTGTTCGTCC] into the high copy number vector pSE280 using
294 standard cloning techniques.

295

296 ***Isolation of Flagellin and Outer membranes***

297 All flagellin and outer membrane (OM) preparations were based on 1 L cultures of strains
298 grown to an OD₆₀₀ of 0.6-0.7. Cells were centrifuged at 3890 *g* and cell pellets resuspended
299 in cold 10mM HEPES pH 7.4. For flagellin isolation, cell suspensions were sheared using
300 an Ultra-Turrax blending stick for 2 minutes set at 13500 rpm. The same protocol was used
301 prior to OM isolation to reduce flagellin contamination. Blended supernatants were
302 centrifuged at 100,000 *g* for 1 hour at 4°C to collect sheared flagellar filaments. The pellets
303 were washed by repeating this procedure three times. Pellets were resuspended in 10mM
304 HEPES pH 7.4 and centrifuged at 3890 *g* to improve the removal of cell debris between
305 each ultra-centrifuge wash step. The washed flagellin pellets were resuspended in 500 µl
306 10mM HEPES pH 7.4 and stored at -20°C.

307

308 For outer membrane isolation cell suspensions were lysed using a Constant Systems cell
309 disruptor at 23kPSI. Lysed cell suspensions were centrifuged at 12000 *g* at 4°C for 40
310 minutes, the supernatant layered onto a sucrose gradient and centrifuged at 56000 *g* for 36
311 hours at 4°C. Outer membrane fractions were resuspended and washed once in 10mM
312 HEPES pH7.4. The washed outer membrane fraction was collected by centrifuging at
313 134000 *g* for 6 hours at 4°C and the resulting pellet resuspended in 500 µl 10mmM HEPES.
314 The quality of preparations was assessed using standard SDS polyacrylamide gel
315 electrophoresis.

316

317 ***NFκB reporter assay, IL-8 ELISA and Immunoblots***

318 Growth conditions for the bladder RT4 cell line has been previously described (26). All
319 challenges were performed using 24 well plates seeded with 500,000 cells in 500 µl, and
320 the cells grown until 80-90% confluent. Bladder RT4 cells were challenged in triplicate with
321 either isolated outer membrane preparations (100 ng/ml protein content), flagellin (0-250
322 ng/ml) or heat-killed whole cells at 37°C and 5% CO₂ (26). Challenges were stopped after
323 24 hours, the extracellular media collected and stored at -20°C. Interleukin 8 concentrations
324 (pg/ml) were assayed using an eBioscience IL-8 ELISA kit following the manufacturer's
325 instructions. Measurement of RT4 NFκB reporter activity was as previously described (26).
326 For IκBα immunoblots, challenged RT4 cells were lysed in RIPA Buffer collected, quantified
327 using a Micro BCA protein assay kit (Thermo) and either stored at -20°C or 10 µg used for
328 immunoblots with an Anti-IκBα antibody (New England Biolabs) (54).

329

330 ***Inhibition of TLR4 and TLR5 siRNA expression***

331

332 TLR4 and TLR5 knockdown experiments using RT4 cells and siRNA were performed as
333 described previously (55). siRNAs used were as follows: s14196 (TLR4) and s14199 (TLR5)
334 and AM4611 (negative siRNA #1) as a control. All challenges were performed as described
335 previously and media bathing the cells analysed using an eBioscience IL-8 ELISA kit
336 following the manufacturer's instructions.

337

338 ***Quantification of Flagellar abundance***

339 Expression of *flgEA240C* was analysed following bacterial growth at 37°C in LB media
340 containing 0.1% arabinose with shaking until an OD600 of 0.6 to 0.7. Staining of the cells
341 was performed using AlexaFluor 568 (20). Essentially, bacterial cell suspensions were
342 immobilised on a 1% agarose padded microscope slide and samples analysed in triplicate
343 at 100x objective using a Nikon Eclipse Ti inverted microscope capturing both phase

344 contrast (100 ms exposure) and red channel images (1000 ms exposure) at five different
 345 fields of view. Five randomly chosen fields were analysed manually using the ImageJ cell
 346 counter plugin generating data where $n = 200 - 300$ cells. Foci per cell was averaged across
 347 the five fields of view to enumerate the level of cell flagellation, as well as the distribution of
 348 flagella over the population.

349

350 ***Data Analysis and Presentation***

351 Data and statistical analysis were performed using MS Excel including the use of ANOVA.
 352 Images for figure panels were processed and cropped using ImageJ and imported into
 353 Adobe illustrator for formatting. All figures were collated in Adobe illustrator to achieve the
 354 correct resolution.

355

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361

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372

373 **Conflict of Interests**

374 The authors declare that there are no conflicts of interest.

375

Figures and Legends

Figure 1: Motility of clinical uro-associated isolates and RT4 bladder cell NF-κB signalling. **A)** Motility agar assay data of a selection of clinical UPEC isolates (Table S1 and S2) showing the diverse range of phenotypes. **B)** Quantification of swarm diameter for n=3 independent colonies of each clinical isolate plotted against NFκB induction. Control strains, NCTC10418 and CFT073, and clinical isolates are colour coded. **C)** NFκB fold induction of all motile and non-motile UPEC clinical isolates. Points have been scattered left or right with respect to the x-axis for clarity.

Figure 2: NF-κB and IL-8 responses of bladder RT4 cells challenged with flagellin, bacterial outer membrane preparations and whole cells. A) NF-κB response of RT4 bladder cells challenged for 24 hours with flagellin filaments (250 ng/ml) isolated from 11 different uro-associated *E. coli* (strains PYL3398-PYL3324: Table S1). **B)** Immunoblot analysis of IκBα following dose dependent challenges of RT4 cells with flagellin filaments isolated from UTI3408. **C)** Quantification of NFκB induction following dose dependent challenges of RT4 cells with flagellin filaments isolated from UTI3408. Data shown is the average of two technical repeats and 3 independent challenges. **D)** IL-8 concentrations of RT4 cell media following transfection with siRNAs targeting either TLR4 or TLR5 expression and challenging for 24 hours with either outer membrane preparations, flagella filaments (50 ng/ml) or heat killed whole bacteria.

401

402

403 **Figure 3: Uro-associated *E. coli* population heterogeneity and UPEC evasion of the**
404 **TLR5 response. A)** Phase contrast and fluorescent images of FlgEA240C foci in the control
405 strains NCTC10418 and CFT073, and two uro-associated clinical isolates. Quantification of
406 a minimum of 250 cells per strain is shown in brackets where Fla: = average number of FlgE
407 foci per cell and Fla⁺: = percentage of the population with foci. Images are chosen to show
408 foci and may not reflect quantified numbers. **B)** Scatter plot showing average foci per cell
409 versus flagellated population. The range of average foci per cell for all strains except
410 NCTC10418 are within experimental error ($P = 0.143$). **C)** Scatter plot showing percentage
411 of flagellated population versus relative NF κ B induction (control strain NCTC10418 = 1.0).
412 **D)** IL-8 production, presented as a ratio, following challenges of RT4 cells with NCTC10418
413 (*pflhDC* -ve) or UTI3408 (*pflhDC* transformed).

414

415

416 **Table S1: Motile Strains and associated data used in this study**

Lab Strain ID	Source*	Flagellin Serotype	Average swarm diameter (cm)	Flagellar abundance §		Reference
				Avg Fla / cell	% Fla ⁺	
Strains used in Figure 1, 2 and 3						
2743	NCTC10418		5.78	2.35	82	(26)
3373	CFT073	H1	7.02	1.59	58	(56)
3398	PYL	H1	3.59	1.46	7	This study
3406	ABU	H18	3.06			
3408	UTI	H1	5.71	1.66	25	
3409	UTI	H5	1.87			
3411	UTI	H6	7.78			
3412	UTI	H18	1.49	1.36	27	
3414	UTI		7.39			
3415	BAC	H5	3.04			
3417	UTI	H1	2.05			
3419	ABU	H6	3.56			
3424	PYL	H18	4.98			
3425	PYL	H1	4.51			
3692	ABU		3.07			
3693	ABU		2.67	1.24	8	
3694	ABU		5.41			
3695	ABU		3.96	1.41	10	
3697	ABU		2.44	1.60	6	
3698	ABU		2.36			
3699	ABU	H4	0.77			
3701	UTI	H27	2.80	1.68	52	
3702	UTI	H4	6.97	1.52	24	
3703	UTI		1.00	1.71	10	
3704	UTI		2.10			
3710	ABU	H4	2.85			
Additional strains used in Figure 3						
4738	ABU	H5	1.58	1.67	50	(8)
4739	ABU	H1	2.41	1.62	35	
4740	ABU	H1	2.25	1.44	57	
4741	ABU	H18	2.10	1.81	11	
4742	ABU	H18	2.25	1.14	19	
4743	ABU	H5	3.67	1.62	36	
4744	ABU	H18	1.67	1.45	46	
4745	ABU	H6	2.00	1.14	7	

417 § Data shown to indicate the strains used in Figure 3

418 *PYL: Pyelonephritis; ABU: asymptomatic bacteriuria; UTI: Acute cystitis; BAC:
419 Bacteraemia

420 **Table S2 Non-motile strains used in Figures 1 and 2D.**

421

Lab Strain ID	Source*
3399	ABU
3400	ABU
3401	UTI
3402	BAC
3403	UTI
3407	UTI
3410	BAC
3413	ABU
3416	ABU
3418	ABU
3420	ABU
3422	PYL
3696	ABU
3701	UTI
3706	UTI
3707	UTI

422

423

*See Table S1 descriptors

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