1 **Evasion of Toll-like Receptor Recognition by *Escherichia coli* is mediated via**

2 **Population Level Regulation of Flagellin Production**

3

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

5

6 **Author Names:** M Lanz[1,*], C Birchall[1,*], L Drage[1,*], D Picton[1,*], C Mowbray[1,*], Q Alsenani[1,*],

7 A. Tan[1], A Ali[1&2], C Harding[2,3], R Pickard[2,3†], J Hall[1,§], PD Aldridge[1,§]

8

9 Addresses:

10 1. Biosciences Institute, Newcastle University, UK
11 2. Translational and Clinical Research Institute, Newcastle University, UK
12 3. Urology Department, Freeman Hospital, Newcastle upon Tyne Hospitals NHS
13   Foundation Trust, UK

14

15 * Contributed equally

16 † Died 2018

17 § co-corresponding authors

18

19 **Key words:** UTI; TLR5; UPEC; Flagellin

20

21 Corresponding author for contact:

22 Phillip D Aldridge

23 phillip.aldridge@ncl.ac.uk

24 Biosciences Institute, Faculty Medical Sciences, Newcastle University, UK

25

26

1

Abstract (300)

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

Introduction

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

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

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

3

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

106 **Results**

107

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

109

110 Forty uro-associated *E. coli* isolates were curated from patients presenting with either

111 cystitis, pyelonephritis, asymptomatic bacteriuria (ABU) or UTI-associated bacteraemia.

112 Semi-quantitative agar assays measuring the size of a bacterial swarm after 8 hrs (Figure

113 1A) were used to assess motility of these isolates and 24 (60%) were identified as motile

114 (Fig 1A & B, Table S1). In general, the swarms of isolates recovered from ABU patients

115 measured between 0.8 and 5.4 cm, while cystitis strains exhibited a bipolar motility

116 distribution pattern with strains swarming less (n=6) or greater (n=4) than 5.4 cm

117 respectively (Fig 1B; P < 0.005).

118

119 The impact of bacterial motility on the urothelial innate response was assessed *in vitro* using

120 heat-killed isolates (1 x 10^5 CFU/ml) and bladder RT4 cells stably transfected with a NFκB

121 luciferase reporter (26). Following these challenges 33 (83%) of all the isolates were

122 associated with a 2-5 fold increase in NFκB signalling (Fig 1B & C), with 7 of the motile

123 isolates associated with increases of >5-fold. Two isolates recovered from cystitis patients

124 were associated with NFκB activities of >30 fold (Fig 1C; P = 0.029) and swarm sizes of >7

125 cm (Fig 1B). These data suggested that bacterial motility linked to a NFκB signalling

126 response, but that the two were not directly correlated.

127

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

129

130 UTIs are ascending infections and urothelial cells respond to potential uropathogens via

131 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 = NS) NFκB 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 IκBα

143 and NFκB 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 IκBα protein levels and

146 NFκB induction was also observed in RT4 urothelial cells (Fig 2B and C). Interestingly, using

147 purified flagelllin 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 NFκB 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 $FlhD_4C_2$ (35). $FlhD_4C_2$ 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

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

10

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 NFκB 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 RflP, a regulator

253   that modulates ClpXP recognition of FlhD$_4$C$_2$, can impact the master regulator FlhD$_4$C$_2$

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 p*flhDC* or

289 pBAD*flgE*A240C 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 p*flhDC* was generated by cloning a PCR product using the primers *flhD*-42FBam

292 [ggcggatccGGGTGCGGCTACGTCGCAC] and *flhC*+616RBam

293   [ggcggatccCGCTGCTGGAGTGTTTGTCC] 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 OD_{600} 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***

14

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% CO_2 (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-IkBa 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.
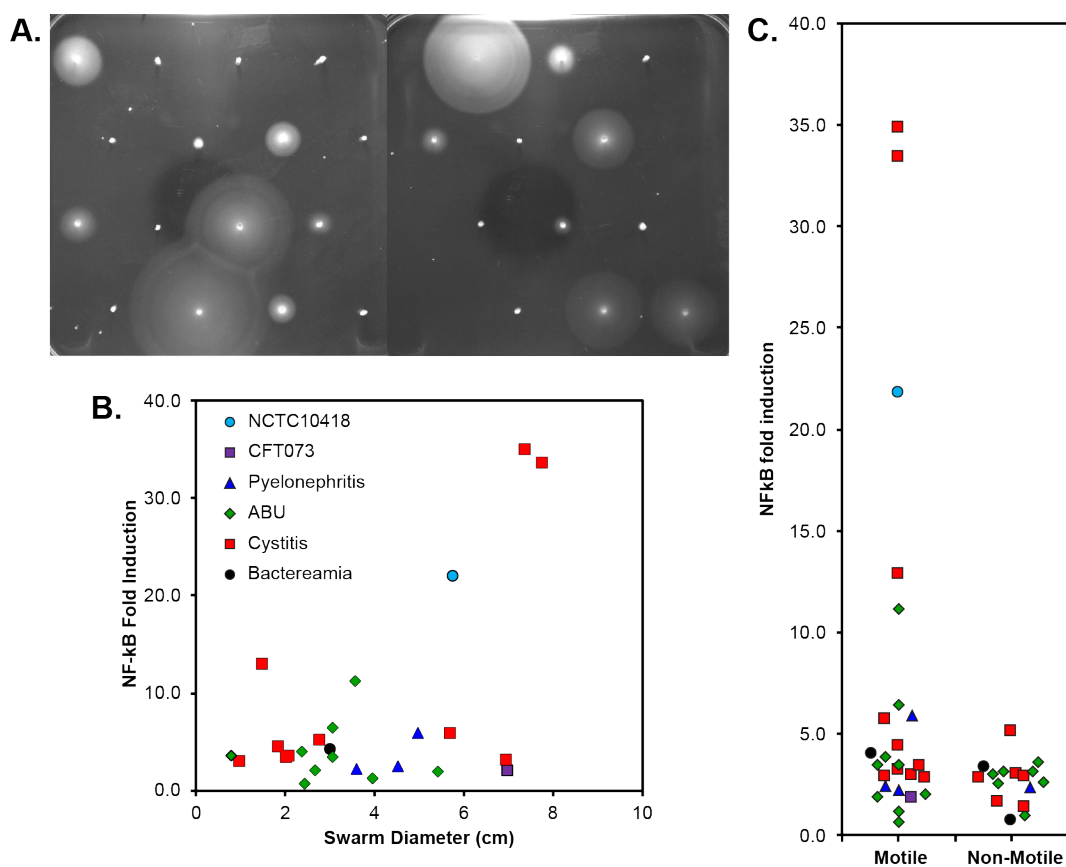
355

Conflict of Interests

The authors declare that there are no conflicts of interest.

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.

387

388
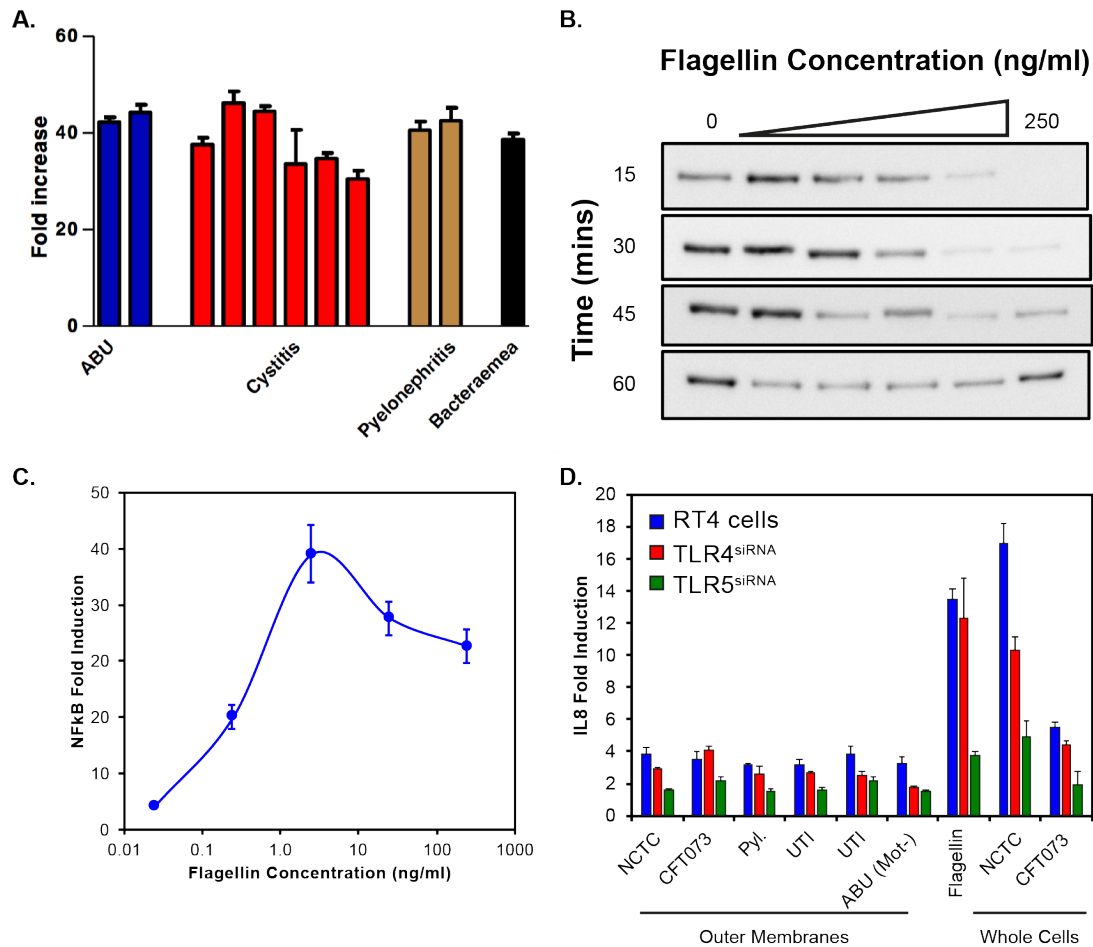
Figure 2: NF-κB and IL-8 responses of bladder RT4 cells challenged with flagellin, bacterial outer membrane preparations and whole cells. A) NF-κB response of RT4 bladder cells challenged for 24 hours with flagellin filaments (250 ng/ml) isolated from 11 different uro-associated *E. coli* (strains PYL3398-PYL3324: Table S1). **B)** Immunoblot analysis of IκBα following dose dependent challenges of RT4 cells with flagellin filaments isolated 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.

400

19

401

402
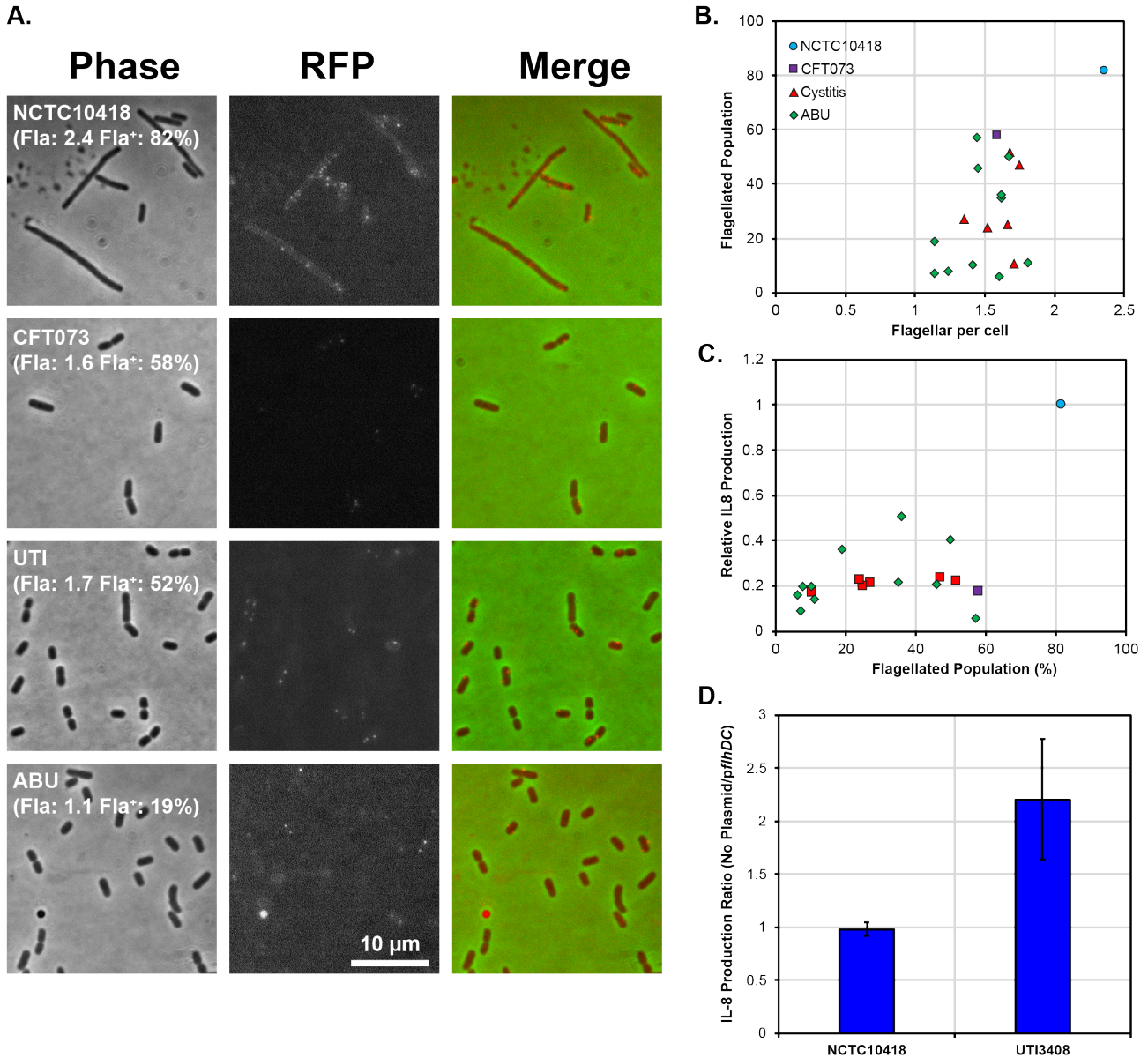
Figure 3: Uro-associated *E. coli* population heterogeneity and UPEC evasion of the TLR5 response. A) Phase contrast and fluorescent images of FlgEA240C foci in the control strains NCTC10418 and CFT073, and two uro-associated clinical isolates. Quantification of a minimum of 250 cells per strain is shown in brackets where Fla: = average number of FlgE foci per cell and Fla+: = percentage of the population with foci. Images are chosen to show foci and may not reflect quantified numbers. **B)** Scatter plot showing average foci per cell versus flagellated population. The range of average foci per cell for all strains except NCTC10418 are within experimental error (P = 0.143). **C)** Scatter plot showing percentage of flagellated population versus relative NFκB induction (control strain NCTC10418 = 1.0). **D)** IL-8 production, presented as a ratio, following challenges of RT4 cells with NCTC10418 (p*flhDC* -ve) or UTI3408 (p*flhDC* 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   *See Table S1 descriptors
423

References

1. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med. 2002 Jul 8;113 Suppl 1A:5S–13S.

2. Klein RD, Hultgren SJ. Urinary tract infections: microbial pathogenesis, host–pathogen interactions and new treatment strategies. Nat Rev Micro. Springer US; 2020 Feb 18;18:211–26.

3. Fisher H, Oluboyede Y, Chadwick T, Abdel-Fattah M, Brennand C, Fader M, et al. Continuous low-dose antibiotic prophylaxis for adults with repeated urinary tract infections (AnTIC): a randomised, open-label trial. Lancet Infect Dis. 2018 Sep;18(9):957–68.

4. Hawn TR, Scholes D, Wang H, Li SS, Stapleton AE, Janer M, et al. Genetic variation of the human urinary tract innate immune response and asymptomatic bacteriuria in women. PLoS ONE. Public Library of Science; 2009;4(12):e8300.

5. Schreiber HL, Conover MS, Chou W-C, Hibbing ME, Manson AL, Dodson KW, et al. Bacterial virulence phenotypes of Escherichia coli and host susceptibility determine risk for urinary tract infections. Science Translational Medicine. American Association for the Advancement of Science; 2017 Mar 22;9(382):eaaf1283.

6. Chan CY, John ALS, Abraham SN. Mast Cell Interleukin-10 Drives Localized Tolerance in Chronic Bladder Infection. Immunity. Elsevier Inc; 2013 Feb 21;38(2):349–59.

7. Choi HW, Bowen SE, Miao Y, Chan CY, Miao EA, Abrink M, et al. Loss of Bladder Epithelium Induced by Cytolytic Mast Cell Granules. Immunity. 2016 Dec;45(6):1258–69.

8. Drage LKL, Robson W, Mowbray C, Ali A, Perry JD, Walton KE, et al. Elevated urine IL-10 concentrations associate with *Escherichia coli* persistence in older patients susceptible to recurrent urinary tract infections. Immun Ageing. Immunity & Ageing; 2019 Jul 10;16:1–11.

9. Lane MC, Alteri CJ, Smith SN, Mobley HLT. Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. Proc Natl Acad Sci USA. 2007 Oct 16;104(42):16669–74.

10. Lane MC, Lockatell V, Monterosso G, Lamphier D, Weinert J, Hebel JR, et al. Role of motility in the colonization of uropathogenic *Escherichia coli* in the urinary tract. Infect Immun. 2005 Nov;73(11):7644–56.

11. Wright KJ, Seed PC, Hultgren SJ. Uropathogenic *Escherichia coli* flagella aid in efficient urinary tract colonization. Infect Immun. 2005 Nov;73(11):7657–68.

12. Chevance FFV, Hughes KT. Coordinating assembly of a bacterial macromolecular machine. Nat Rev Micro. 2008 Jun;6(6):455–65.

13. Minamino T, Imada K. The bacterial flagellar motor and its structural diversity. Trends Microbiol. 2015 May;23(5):267–74.

14. Minamino T. Protein export through the bacterial flagellar type III export pathway. Biochim Biophys Acta. 2014 Aug;1843(8):1642–8.

15. Porter SL, Wadhams GH, Armitage JP. Signal processing in complex chemotaxis pathways. Nat Rev Micro. Nature Publishing Group; 2011 Feb 1;9(3):153–65.

16. Smith TG, Hoover TR. Deciphering bacterial flagellar gene regulatory networks in the genomic era. Adv Appl Microbiol. 2009;67:257–95.

17. Snyder LAS, Loman NJ, Fütterer K, Pallen MJ. Bacterial flagellar diversity and evolution: seek simplicity and distrust it? Trends Microbiol. 2009 Jan;17(1):1–5.

18. Aldridge P, Hughes KT. Regulation of flagellar assembly. Curr Opin Microbiol. 2002 Apr;5(2):160–5.

19. Chilcott GS, Hughes KT. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Microbiol Mol Biol Rev. 2000 Dec;64(4):694–708.

20. Sim M, Koirala S, Picton D, Strahl H, Hoskisson PA, Rao CV, et al. Growth rate control of flagellar assembly in *Escherichia coli* strain RP437. Sci Rep. 2017 Jan 24;7:41189.

21. Andersen-Nissen E, Hawn TR, Smith KD, Nachman A, Lampano AE, Uematsu S, et al. Cutting edge: Tlr5-/- mice are more susceptible to *Escherichia coli* urinary tract infection. J Immunol. 2007 Apr 15;178(8):4717–20.

22. Ali ASM, Townes CL, Hall J, Pickard RS. Maintaining a sterile urinary tract: the role of antimicrobial peptides. J Urol. 2009 Jul;182(1):21–8.

23. Sivick KE, Mobley HLT. Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. Infect Immun. 2010 Feb;78(2):568–85.

24. Herrmann B, Burman LG. Pathogenesis of *Escherichia coli* cystitis and pyelonephritis: apparent lack of significance of bacterial motility and chemotaxis towards human urine. Infection. 1985 Jan;13(1):4–7.

25. Schaeffer AJ, Schwan WR, Hultgren SJ, Duncan JL. Relationship of type 1 pilus expression in *Escherichia coli* to ascending urinary tract infections in mice. Infect Immun. American Society for Microbiology (ASM); 1987 Feb;55(2):373–80.

26. Ali ASM, Mowbray C, Lanz M, Stanton A, Bowen S, Varley CL, et al. Targeting Deficiencies in the TLR5 Mediated Vaginal Response to Treat Female Recurrent Urinary Tract Infection. Sci Rep. Nature Publishing Group; 2017 Sep 8;7(1):11039.

27. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SLR, Cookson BT, Logan SM, et al. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci USA. 2005 Jun 28;102(26):9247–52.

28. Wang L, Rothemund D, Curd H, Reeves PR. Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. J Bacteriol. 2003 May;185(9):2936–43.

500   29.   Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate
501   immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature.
502   2001 Apr 26;410(6832):1099–103.

503   30.   Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SLR, et
504   al. Toll-like receptor 5 recognizes a conserved site on flagellin required for
505   protofilament formation and bacterial motility. Nat Immunol. 2003 Dec;4(12):1247–
506   53.

507   31.   Samuelsson P, Hang L, Wullt B, Irjala H, Svanborg C. Toll-like receptor 4 expression
508   and cytokine responses in the human urinary tract mucosa. Infect Immun. American
509   Society for Microbiology Journals; 2004 Jun;72(6):3179–86.

510   32.   Ragnarsdottir B, Samuelsson M, Gustafsson MCU, Leijonhufvud I, Karpman D,
511   Svanborg C. Reduced toll-like receptor 4 expression in children with asymptomatic
512   bacteriuria. J Infect Dis. Oxford University Press; 2007 Aug 1;196(3):475–84.

513   33.   Song J, Bishop BL, Li G, Grady R, Stapleton A, Abraham SN. TLR4-mediated
514   expulsion of bacteria from infected bladder epithelial cells. Proc Natl Acad Sci USA.
515   2009 Sep 1;106(35):14966–71.

516   34.   Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-Like Receptor 4, But
517   Not Toll-Like Receptor 2, Is a Signaling Receptor for Escherichia and Salmonella
518   Lipopolysaccharides. The Journal of Immunology. 2000 Nov 15;165(10):5780–7.

519   35.   Wang S, Fleming RT, Westbrook EM, Matsumura P, McKay DB. Structure of the
520   *Escherichia coli* FlhDC complex, a prokaryotic heteromeric regulator of transcription.
521   Journal of Molecular Biology. 2006 Jan 27;355(4):798–808.

522   36.   Claret L, Hughes C. Rapid Turnover of FlhD and FlhC, the Flagellar Regulon
523   Transcriptional Activator Proteins, during Proteus Swarming. J Bacteriol. 2000 Feb
524   1;182(3):833–6.

525   37.   Soutourina OA, Bertin PN. Regulation cascade of flagellar expression in Gram-
526   negative bacteria. FEMS Microbiol Rev. 2003 Oct 1;27(4):505–23.

527   38.   Kitagawa R, Takaya A, Yamamoto T. Dual regulatory pathways of flagellar gene
528   expression by ClpXP protease in enterohaemorrhagic *Escherichia coli.* Microbiology.
529   2011 Nov 2;157(11):3094–103.

530   39.   Takaya A, Erhardt M, Karata K, Winterberg K, Yamamoto T, Hughes KT. YdiV: a
531   dual function protein that targets FlhDC for ClpXP-dependent degradation by
532   promoting release of DNA-bound FlhDC complex. Mol Microbiol. 2012 Mar
533   2;83(6):1268–84.

534   40.   Josenhans C, Suerbaum S. The role of motility as a virulence factor in bacteria. Int J
535   Med Microbiol. 2002 Mar;291(8):605–14.

536   41.   Veening J-W, Smits WK, Kuipers OP. Bistability, epigenetics, and bet-hedging in
537   bacteria. Annu Rev Microbiol. 2008;62:193–210.

538   42.   Mears PJ, Koirala S, Rao CV, Golding I, Chemla YR. *Escherichia coli* swimming is
539   robust against variations in flagellar number. Elife. 2014;3:e01916.

43. Brown J, Faulds-Pain A, Aldridge P. The coordination of flagellar gene expression and the flagellar assembly pathway. In: Pili and Flagella: Current Research and Future Trends. 2009. pp. 99–120.

44. Koirala S, Mears P, Sim M, Golding I, Chemla YR, Aldridge PD, et al. A Nutrient-Tunable Bistable Switch Controls Motility in *Salmonella enterica* Serovar Typhimurium. MBio. 2014;5(5).

45. Jenal U, Stephens C. The *Caulobacter* cell cycle: timing, spatial organization and checkpoints. Curr Opin Microbiol. 2002 Dec 1;5(6):558–63.

46. Alteri CJ, Smith SN, Mobley HLT. Fitness of *Escherichia coli* during urinary tract infection requires gluconeogenesis and the TCA cycle. PLoS Pathog. 2009 May;5(5):e1000448.

47. Forsyth VS, Armbruster CE, Smith SN, Pirani A, Springman AC, Walters MS, et al. Rapid Growth of Uropathogenic *Escherichia coli* during Human Urinary Tract Infection. MBio. 2018 Mar 6;9(2).

48. Sintsova A, Frick-Cheng AE, Smith S, Pirani A, Subashchandrabose S, Snitkin ES, et al. Genetically diverse uropathogenic *Escherichia coli* adopt a common transcriptional program in patients with UTIs. Elife. 2019 Oct 21;8.

49. Wada T, Hatamoto Y, Kutsukake K. Functional and expressional analyses of the anti-FlhD4C2 factor gene *ydiV* in *Escherichia coli*. Microbiology. 2012 Jun 1;158(Pt_6):1533–42.

50. Reitzer L, Zimmern P. Rapid Growth and Metabolism of Uropathogenic *Escherichia coli* in Relation to Urine Composition. Clin Microbiol Rev. 2019 Dec 18;33(1).

51. Spurbeck RR, Alteri CJ, Himpsl SD, Mobley HLT. The Multifunctional Protein, YdiV, Represses P Fimbriae-Mediated Adherence in Uropathogenic *Escherichia coli.* J Bacteriol. 2013 May 10.

52. Stewart MK, Cookson BT. Non-genetic diversity shapes infectious capacity and host resistance. Trends Microbiol. 2012 Oct;20(10):461–6.

53. Casadesús J, Low DA. Programmed Heterogeneity: Epigenetic Mechanisms in Bacteria. Journal of Biological Chemistry. 2013 May 17;288(20):13929–35.

54. Aldridge P, Jenal U. Cell cycle-dependent degradation of a flagellar motor component requires a novel-type response regulator. Mol Microbiol. 1999 Apr;32(2):379–91.

55. Mowbray CA, Shams S, Chung G, Stanton A, Aldridge P, Suchenko A, et al. High molecular weight hyaluronic acid: a two-pronged protectant against infection of the urogenital tract? Clin Transl Immunol. 2018 Jun 7;7(6):e1021–11.

56. Brzuszkiewicz E, Brüggemann H, Liesegang H, Emmerth M, Olschläger T, Nagy G, et al. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. Proc Natl Acad Sci USA. 2006 Aug 22;103(34):12879–84.