

1 **TITLE: EFFECTS OF A MUTATION IN THE *gyrA* GENE ON THE**
2 **VIRULENCE OF UROPATHOGENIC *Escherichia coli***

3 **Running title:** *gyrA* gene and virulence in UPEC

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25 **ABSTRACT**

26 Fluoroquinolones are among the drugs most extensively used for the treatment of
27 bacterial infections both in human and veterinary medicine. Resistance to quinolones
28 can be both chromosomal- and plasmid-mediated. The former mechanism is associated
29 with mutations in the DNA gyrase and topoisomerase IV encoding genes as well as
30 mutations in regulatory genes affecting different efflux systems, among others. We
31 studied the role of the acquisition of a mutation in the *gyrA* gene in the virulence and
32 protein expression of uropathogenic *E. coli*. The HC14366M strain carrying a mutation
33 in the *gyrA* gene (S83L) was found to lose the capacity to cause both cystitis and
34 pyelonephritis mainly due to a decrease in the expression of the *fimA*, *papA*, *papB* and
35 *ompA* genes. The levels of expression of the *fimA*, *papB* and *ompA* genes were
36 recovered on complementing the strain with a plasmid containing the *gyrA* wild-type
37 gene. However, only a slight recovery was observed in the colonization of the bladder in
38 the GyrA complement strain compared to the mutant strain in a murine model of
39 ascending urinary tract infection. In conclusion, a mutation in the *gyrA* gene of
40 uropathogenic *E. coli* reduced the virulence of the bacteria likely in association with the
41 effect of DNA supercoiling on the expression of several virulence factors and proteins,
42 thereby decreasing their capacity to cause cystitis and pyelonephritis.

43

44 **INTRODUCTION**

45 Fluoroquinolones are among the drugs most extensively used for the treatment of
46 bacterial infections both in human and veterinary medicine. They act by inhibiting the
47 DNA gyrase and topoisomerase IV which tetrameric enzymes constituted by two A
48 subunits and two B subunits. These subunits are encoded by the *gyrA* and *gyrB* genes,
49 respectively, in the case of the DNA-gyrase and by the *parC* and *parE* genes,
50 respectively, in the case of topoisomerase IV (1). The quinolones bind the DNA and the
51 topoisomerase forming a quinolone-DNA-topoisomerase complex, avoiding the
52 transcription or replication of DNA (1). The main mechanism of quinolone resistance is
53 the accumulation of mutations in these two enzymes (2). Quinolone resistance can also
54 be caused by the acquisition of *qnr*, a plasmid-mediated horizontally transferable gene
55 (3). Two additional plasmid-mediated mechanisms of resistance to quinolones have also
56 been identified, the AAC(6')-Ib-cr protein, a variant aminoglycoside acetyltransferase
57 capable of reducing ciprofloxacin activity (4), and the efflux pump QepA (5).

58

59 The primary cellular target of fluoroquinolones in *E. coli* is a type II topoisomerase
60 (DNA gyrase) enzyme which is unique in catalyzing negative supercoiling of covalently
61 closed circular double-stranded DNA in an ATP-consuming reaction and is therefore
62 essential for maintenance of DNA topology. Topoisomerase IV has been shown to be a
63 secondary quinolone target in *E. coli* and decatenates the chromosome before cell
64 division (6). Changes in DNA supercoiling in response to environmental factors
65 contribute to the control of bacterial virulence (7).

66 Quinolone- and fluoroquinolone-resistant uropathogenic *E. coli* (UPEC) strains display
67 reduced virulence in the invasion of immunocompromised patients. By contrast,
68 susceptible *E. coli* strains are more virulent and affect immunocompetent hosts,

69 showing a larger number of virulence factors contained in pathogenicity islands (PAIs)
70 (8, 9). It has been demonstrated that a resistant *E. coli* strain becomes less virulent
71 following the acquisition of a *gyrA* mutation (10), and that the loss of virulence by
72 acquisition of quinolone resistance may take place before the acquisition of mutations
73 and/or quinolone resistance levels (11).

74 The biological cost of quinolone resistance differs among different bacteria and depends
75 on the level of resistance and the number of resistance mutations (12).

76 In comparison to commensal strains UPEC has several virulence factors that allow it to
77 colonize host mucosal surfaces, injure and invade host tissues, overcome host defense
78 mechanisms and incite a host inflammatory response.

79 Among these virulence factors, type 1 fimbriae, P-fimbriae and outer membrane
80 proteins have an important role in several steps of urinary tract infection (UTI). Thus,
81 type 1 pili promote adherence of UPEC to superficial bladder epithelial cells initiating a
82 cascade of events that directly influence the pathogenesis of UTIs (13). In addition, type
83 1 fimbriae have been associated with invasion of the bladder epithelial cells and the
84 ability of the bacteria to replicate intra-cellularly, forming “internal biofilms” (14).

85 P fimbria (a mannose-resistant adhesin of UPEC) has been shown to be associated with
86 acute pyelonephritis (at least 90% of acute pyelonephritis) (15).

87 On the other hand, the OmpA protein is critical for promoting persistent infection
88 within the epithelium and has been associated with cystitis and intracellular survival
89 (16).

90

91 The aim of this study was to determine the role of the acquisition of a mutation in the
92 *gyrA* gene in the virulence and protein expression of UPEC.

93

94 **MATERIAL AND METHODS**

95 **Bacterial strains and selection of resistant mutants.** Three strains of *E. coli* were
96 used in this study: i) the HC14366 wild-type UPEC clinical isolate with a MIC of
97 ciprofloxacin (CIP) of 0.008 mg/L; ii) its CIP-resistant mutant (*E. coli* HC14366M)
98 with a mutation in the *gyrA* gene (S83L) and a MIC of CIP of 2 mg/L; and iii) the *E.*
99 *coli* HC14366M mutant transformed with a plasmid carrying the wild-type *gyrA* gene,
100 generating a complementation of the *gyrA* gene (*E. coli* HC14366MC) with a MIC of
101 CIP of 0.064 mg/L. Strain HC14366-wt was grown at 37°C on MacConkey plates in the
102 presence of ciprofloxacin in a multi-step selection process to obtain strain HC14366-2
103 (HC14366M), a ciprofloxacin-resistant mutant. Ciprofloxacin (Fluka, Steinheim,
104 Germany) was only present in agar plates during the selection procedures, starting at
105 0.004 mg/L (half of the MIC for HC14366-wt) and increasing 2-fold each step, until
106 reaching a maximum concentration of 2 mg/L. Single colonies were selected at each
107 step and named according to the ciprofloxacin concentration of selection (e.g., strain
108 HC14366-0.016 was selected at a CIP concentration of 0.016 µg/mL).

109

110 **Antimicrobial susceptibility.** Susceptibility to several antimicrobial agents was
111 determined in the presence and absence of 20 mg/L of the efflux pump inhibitor Phe-
112 Arg-β-naβhthylamide using the agar dilution method according to the CSLI (17)
113 guidelines as described elsewhere (18).

114

115 **Virulence profile.** The virulence profile was analyzed by PCR using gene-specific
116 primers for 17 virulence genes including hemolysin (*hly*), cytotoxic necrotizing factor
117 (*cnf*), autotransporter (*sat*), P-fimbriae (*pap* genes), type 1C fimbriae (*foc*),
118 yersiniabactin (*fyu*), heat-resistant hemagglutinin (*hra*), S-fimbriae (*sfa*), invasins (*ibeA*),

119 adhesin (*iha*), aerobactin (*aer*), siderophores (*iucC*, *iutA*, *iroN*), and antigen 43 (*ag43*)
120 (19).

121

122 **Motility and type 1 fimbriae expression.** The motility of each isolate was analyzed by
123 growth in mannitol agar. Expression of type 1 fimbriae was determined by agglutination
124 of *Saccharomyces cerevisiae* by the procedure described by (20).

125

126 **Doubling time analysis.** The strains were grown in LB media at 37°C with shaking.
127 The OD_{600 nm} of each culture was measured in a CECIL CE2302 spectrum. Aliquotes
128 were taken every 30 minutes along six hours (21).

129

130 **Animal model.** The virulence of the strains was tested in a murine model of an
131 ascending UTI protocol approved by the Danish Ministry of Justice Animal Ethics
132 Committee (approval no. 2004/561-835) and described by (22). In short, mouse
133 bladders were emptied by gently pressing the abdomen, and 50 µl (5×10^6 CFU) of
134 each bacterial suspension was slowly inoculated transurethrally into 4 to 6 outbred
135 female albino CFW-1 mice (26 to 30 g; Harlan Netherlands, Horst, Netherlands) with
136 the use of plastic catheters. The mice were housed 4 to 6 to a cage and were given free
137 access to food and 5% glucose-containing water. Seventy-two hours after inoculation,
138 urine was collected from each mouse. The mice were then euthanized by cervical
139 dislocation, and the bladder and kidneys were removed and stored in Eppendorf tubes.
140 The urine samples were processed the same day by spotting (20 µl) of a series of 10-
141 fold dilutions (10^0 to 10^{-6}) in duplicate on bromothymol blue agar plates (SSI
142 Diagnostika, Hillerød, Denmark). The bladder and kidneys were stored in 0.9% saline
143 solution and were then incubated at room temperature for 1 h and subsequently

144 homogenized using a TissueLyser (Qiagen, Ballerup, Denmark). Plates for bacterial
145 counting were processed as described above. The detection limit was 25 CFU/sample.
146 The experiment was repeated twice. The three strains were tested in parallel on the same
147 day and using the same batch of mice.

148

149 **RT-PCR.** The strains were grown to an OD_{620nm} of 0.5 in Luria-Bertani medium. One
150 ml was centrifuged and RNA from the pellet was extracted with TriReagent solution
151 (Ambion, Spain) following the manufacturer's instructions, and treated with 1 µl of
152 DNA-free DNase (Ambion, Spain). RT-PCR was performed using the AccessQuick
153 RT-PCR System (Promega, Spain). Five hundred nanograms of RNA were taken as
154 template. Specific primers were used for the housekeeping *gap* gene (used as an
155 expression control) (5'-GTATCAACGGTTTTGGCCG-3'/5'-AGCTTTAGCAGCA
156 CCGGTA-3') generating an amplicon of about 550 bp; the *fimA* gene
157 (GGACAGGTTCGTACCGCATC/ACGTTGGTATGACCCGCATC) generating an
158 amplicon of about 250 bp; the *marA* gene (CATTCATAGCTTTTGGACTGGAT/GTG
159 TAAAAAGCGCGATTCGCC) generating an amplicon of about 150 bp; the *papA* gene
160 (GGGGCAGGGTAAAGTAACTT/CAGGGTATTAGCATCACCT); and the *papI* gene
161 (CGATGAGTGAATATATGAA/CACGAATTCTTATTAAGTTGTGGAAGA).The
162 PCR reaction was performed under the following conditions: one cycle of 45 minutes at
163 45°C and 3 minutes at 94°C, followed by 26-28 cycles (*fimA*, *marA*, *papA* and *papI*
164 genes) or 16 cycles (*gap* gene) of 1 minute at 94°C, 1 minute at 56°C, and 1 minute at
165 72°C. The PCR products were run in commercial acrylamide gels (GeneGel Excel, GE
166 Healthcare, Spain) and stained with the Plus One DNA Silver staining kit (GE
167 Healthcare, Spain). All experiments were carried out in triplicate.

168

169 **Protein analysis.** Purification of whole proteins was performed using a sonicator-based
170 method (23). Two-dimensional gels electrophoresis was run for the protein extracts of
171 these three strains and stained using a silver staining protocol to compare their patterns.
172 The spots in the HC14366 wild-type *E. coli* showing a variation in the level of
173 abundance compared to the mutant strain (*E. coli* HC14366M) and restored in the
174 transformed *E. coli* (*E. coli* HC14366MC), were sliced and characterized by mass
175 spectrometry analysis (MALDI TOF-TOF).

176

177 **Real-time experiments.** RNA was extracted from exponential cultures and isolated
178 using RNeasy Protect Bacteria Reagent (Qiagen, Hilden, Germany) and the RNeasy Mini
179 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All
180 samples were treated with the DNA-free DNase kit (Ambion, Austin, TX) to remove
181 contamination by genomic DNA, and a PCR was performed to confirm the loss of
182 DNA. In this step, quantification of the RNA was carried out by EPOCH (Biotek).
183 Three independent RNA extractions of each sample were performed. Using the retro-
184 transcription kit (Takara Cat#RR037Q), 500 ng of each RNA sample were used to
185 perform reverse transcription. The cDNA template was diluted 1/5 for the RT-PCR. The
186 *ompA*, *ompF* (both encoding two outer membrane proteins related to virulence), and
187 *papB* (one of the transcription regulators of *papA*) genes were selected and the *16S* gene
188 was used as an endogenous control. Primer Express® software was used to design the
189 primers to amplify these genes. After several assays with different primer
190 concentrations, a concentration of 3 μM was found to be optimal. Amplification was
191 performed using a StepOne™ Real-Time PCR System (Applied Biosystems) using the
192 Sybr Premix Ex Taq™ "Tli RNaseH Plus" kit (Takara) and the Universal Thermal
193 Cycling conditions: 2 min at 50°C (UNG activation), 10 min at 95°C (enzyme

194 activation) followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C
195 (anneal/extension) for 1 min. Data was analyzed with the StepOne software v2.0 and the
196 relative level of expression of each sample ($2^{-\Delta\Delta CT}$) was obtained.

197

198 **Statistical analysis.** Data from the animal model experiments was analyzed using the
199 one factor ANOVA with the SPSS software version 20. P-values less than 0.05 were
200 considered to be significant.

201

202 **RESULTS**

203

204 The HC14366 UPEC strain was submitted to a multi-step selection process in the
205 presence of CIP, starting at 0.004 mg/L (half of the MIC for the wild-type strain) and
206 increasing 2-fold each step until reaching a maximum concentration of 5.12 mg/L. The
207 intermediate mutant HC14366-2.56 (HC14366M) was chosen because it has a mutation
208 in the QRDR of the *gyrA* gene but not in the *gyrB*, *parC* or *parE* genes. This mutation is
209 found in codon 83 from Ser to Leu.

210 The HC14366M mutant was transformed with a plasmid carrying the wild-type *gyrA*
211 gene. The resulting strain (HC14366MC) showed a MIC to CIP of 0.064 mg/L. The
212 MICs of different antimicrobial agents in the presence/absence of the efflux pump
213 inhibitor Phe-Arg- β -naphthylamide were also determined (Table 1). The complemented
214 strain HC14366MC was found to be less resistant to CIP, nalidixic acid, norfloxacin and
215 chloramphenicol than the mutant strain HC14366M.

216 The HC14366 wild-type strain and its mutants showed the following virulence factors:
217 hemolysin (*hly*), cytotoxic necrotizing factor (*cnf1*), autotransporter (*sat*), yersiniobactin
218 (*fyuA*), type 1 fimbriae (*fimA*), P-fimbriae (*pap* genes), hemagglutinin (*hra* gene), S-
219 fimbriae (*sfaS*), and siderophore (*iroN*). The HC14366M and HC14366MC strains
220 showed a decrease in the motility through mannitol and in the expression of type 1
221 fimbriae in comparison with the wild-type strain. Therefore, expression of type 1
222 fimbriae and motility are not affected by a mutation in the *gyrA* gene.

223 The doubling time of the three strains was studied, showing that a mutation in the *gyrA*
224 gene affects bacterial growth, and the complemented strain showed a higher doubling
225 time value than the mutant strain but could not fully recover the wild-type levels (data
226 not shown).

227 These three strains were inoculated into six mice of an animal model of ascending UTI,
228 and urine, bladder and kidney samples were collected. It is noteworthy that the
229 HC14366M strain lost the capacity to cause cystitis and pyelonephritis, with an average
230 of 10^5 CFU/ml, 10^2 CFU and 10^0 CFU found in urine, the bladder and the kidneys,
231 respectively, compared with the values observed in the wild-type strain: 10^8 CFU/ml
232 urine ($p= 0.032$), 10^7 CFU/bladder ($p= 0.002$) and 10^4 CFU/two kidneys ($p= 0.042$).
233 The HC14366MC strain increased the capacity to cause cystitis showing around 10^4 ($p=$
234 0.011) CFU in the bladder but did not have the capacity to cause pyelonephritis ($p=$
235 0.043) (Fig. 1).

236 In order to determine the cause of the decrease of colonization in the mutant strain, RT-
237 PCR were carried out using specific primers for the *fimA* and *papA* genes involved in
238 cystitis and pyelonephritis, respectively. The expression of both genes was found to be
239 decreased in the HC14366M strain and only *fimA* expression was recovered in the
240 complemented strain. On the other hand, *marA* was overexpressed in the mutant and
241 complemented strains in comparison with the wild-type strain (Fig. 2).

242 In order to study the cause of the decrease in the expression of the *papA* gene in both the
243 mutant and complemented strain, we studied the regulators Lrp, PapI and PapB. A total
244 inhibition of *papB* and *papI* gene expression was found in the HC14366M strain, being
245 *papB* expression recovered in the HC14366MC strain (Fig. 4).

246 Protein analysis revealed changes in protein expression in the three strains (Table 2, Fig.
247 3). These changes included proteins implicated in cellular permeability, metabolic
248 functions and DNA replication. Among the proteins with decreased expression in the
249 HC14366M strain but with the recovery of wild-type levels in the HC14366MC strain
250 we found the outer membrane protein A precursor, aspartate ammonia-lyase, the
251 maltose-binding periplasmic protein, tryptophanyl-tRNA synthetase, the D-ribose

252 periplasmic binding protein, the pyruvate kinase I protein, and a phosphate
253 acetyltransferase. On the other hand, the DNA-directed RNA polymerase, two
254 dehydrogenases and the heat shock protein Hsp90 were overexpressed in the
255 HC14366M but not in the HC14366wt or HC14366MC strains. In addition, the
256 expression of the outer membrane protein F (porin) decreased in the HC14366M and its
257 complemented strain (Table 2).

258 RNA expression of the genes encoding some proteins possibly related to virulence
259 (MalE, OmpA, OmpF, and PapB) was analyzed confirming the data obtained in the
260 protein experiments (Fig. 4).

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268 **DISCUSSION**

269 Since their introduction into clinical use in 1983, fluoroquinolones have played an
270 essential role in the treatment of infectious diseases caused by enteric bacteria such as
271 *E. coli*. However, a progressive increase in the emergence of fluoroquinolone-resistant
272 strains has been observed in the last decades (24). Two types of mutants are
273 predominantly found among clinical isolates: low-level resistant isolates (CIP MIC<2
274 mg/L) frequently carrying a single *gyrA* mutation which generates a substitution of
275 serine 83 to leucine (S83L) and high-level resistant isolates (MIC >4 mg/L) carrying
276 two *gyrA* mutations in addition to mutations affecting serine 80 (S80) and glutamic acid
277 84 (Glu84) in *parC* (21).

278 The “in vitro” mutant obtained in our laboratory presented the single *gyrA* mutation
279 most frequently found in clinical isolates (S83L).

280 The level of global supercoiling in *E. coli*, is mainly regulated by the DNA-gyrase (25).
281 The accumulation of mutations in genes which encode for the essential enzymes
282 involved in the control of DNA topology can affect the regulation of the degree of
283 supercoiling. Thus, the expression of supercoiling-regulated genes in laboratory mutants
284 is commonly associated with a fitness cost (probably due to the overexpression of an
285 unknown efflux system), observed as a reduced growth rate and/or virulence in the
286 absence of antibiotic (21). In accordance with the results obtained in our study, Bagel et
287 al. (21) observed that a single S83L mutation in the *gyrA* gene showed an increase in the
288 doubling time and, therefore, a decrease in the growth rate in comparison with the wild-
289 type strain. Moreover, in the present study, an increase was observed in the doubling
290 time when plasmid encoded *gyrA*⁺ was introduced into mutant strain, albeit not to wild-
291 type levels. These results indicate that *gyrA* is involved in the rate of *E. coli* growth.

292

293 Changes in DNA supercoiling affect antimicrobial resistance levels. Thus, the
294 introduction of a plasmid-encoded allele of *gyrA*⁺ in the HC14366M strain caused a
295 reduction in the MICs of CIP and nalidixic acid (from 2.56 to 0.064 mg/L and from
296 >256 to 6 mg/L, respectively), indicating that this mutation contributes to the expression
297 of quinolone resistance as described previously (21).

298 Changes in DNA supercoiling can also contribute to the control of bacterial virulence
299 (7). The mutation in the *gyrA* gene in the strain under study seemed to cause changes in
300 its capacity to develop cystitis and pyelonephritis. Firstly, a reduction in type 1 fimbriae
301 expression was shown by the mutant strain, preventing it from colonizing the bladder
302 and, therefore, from causing cystitis. The finding that the introduction of a plasmid-
303 encoded *gyrA*⁺ did not significantly ($p = 0.456$) modify the capacity of the mutant strain
304 to cause cystitis could be due to the fact that transcription from the *fimA* promoter was
305 not totally affected by changes in DNA supercoiling as demonstrated by Dove et al. (26)
306 on introducing a *topA:Tn10* mutation or inhibiting the DNA-gyrase with the antibiotic
307 novobiocin.

308 Another change in virulence as a consequence of the acquisition of a mutation in the
309 *gyrA* gene is a decrease in P-fimbriae expression leading a decrease in the capacity of
310 the mutant strain to cause pyelonephritis. Expression of pyelonephritis-associated pili
311 (Pap) in *E. coli* is under a phase-variation control mechanism in which individual cells
312 alternate between pili⁺ (ON) and pili⁻ (OFF) states through a process involving DNA
313 methylation by deoxyadenosine methylase (Dam) and regulation via Lrp (27).

314 Control of P-fimbriae expression also requires the action of PapI, a positive regulator
315 that increases the affinity of Lrp for the binding sites, and PapB, the second specific
316 regulator of the Pap operon, that plays an important role at a transcriptional level
317 primarily by coordinating the expression of *papBA* and *papI* promoters (28).

318 In our strain, Lrp and PapI seem to be functional. However, a decrease in *papB* and *papI*
319 expression was found in the mutant strain, with only *papB* expression being recovered
320 in the complemented strain, albeit not at wild-type levels, and not being reflected in the
321 ability of the complemented strain to colonize the kidney.

322 Tessier MC et al. (29) studied F165 adhesin from *E. coli*. This adhesin belongs to the
323 family of Pap-related fimbriae the expression of which is mediated by regulatory
324 proteins such as Lrp, Dam-methylase, and by FooI and FooB. They found that
325 inactivation of the *gyrA* gene caused a decrease in supercoiling producing a decrease in
326 *fooB* expression and inducing a decrease in P-fimbriae expression. FooB is the
327 equivalent of PapB in the P-fimbriae. The decrease of *papB* expression found in the
328 present study could explain the decrease of P-fimbriae expression, thereby making the
329 mutant strain unable to adhere to renal epithelial cells and cause pyelonephritis.

330 Although *papB* expression was recovered in the complemented strain, the finding that it
331 did not recover the ability to cause pyelonephritis may be due to the fact that other P-
332 fimbriae regulator (as PapI) were not affected by the inclusion of the plasmid containing
333 the functional *gyrA* gene.

334 Finally, the introduction of a mutation in the *gyrA* gene may cause changes in the
335 expression of different proteins.

336 Treatment with fluoroquinolones can induce heat shock responses (30). For example,
337 levofloxacin produced an overexpression of several heat shock proteins when the strain
338 was incubated with this antibiotic (30), being HtpG one of these proteins. HtpG is the
339 bacterial homologue of Hsp90 (presented in yeast and humans) and is dispensable under
340 non-stress conditions. HtpG comprises a large fraction (0.36%) of all the proteins in *E.*
341 *coli* growing at 37°C (31). In the present study, this protein was found to be
342 overexpressed in the mutant strain and its expression achieved wild-type levels in the

343 strain complemented with the plasmid-encoded *gyrA*⁺. Therefore, the transcription of
344 HtpG is mainly regulated by supercoiling.

345 OmpA is a major, monomeric, integral protein component of the outer bacterial
346 membrane that functions as a critical determinant of intracellular virulence for UPEC,
347 promoting persistent infection within the bladder epithelium (32). The fact that the
348 HC14366M strain has a significantly lower bladder colonization rate than the HC14366
349 wild-type strain may be in accordance with the decrease in the expression of this gene.
350 The recovery of *ompA* expression together with that of *fimA* could explain the increase
351 in bladder colonization from 10² CFU/g to 10⁴ CFU/g.

352 OmpF is also one of the major outer membrane proteins of *E. coli*, the expression of
353 which is extremely and specifically sensitive to the level of DNA supercoiling (33). Our
354 results are in accordance with the study by Graene-Cook et al. (33) in which the finding
355 of a *gyrA* mutant strain led to a decrease in OmpF expression probably due to
356 overexpression of *marA* (34).

357

358

359 In conclusion, a mutation in the *gyrA* gene of UPEC causes a decrease in the virulence
360 of the bacteria due to the effect of DNA supercoiling on the expression of several
361 virulence factors and proteins, thereby decreasing the capacity to cause cystitis and
362 pyelonephritis. This study demonstrates the relationship between virulence and the
363 acquisition of antimicrobial resistance “in vivo”.

364

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366

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502 **Figure 1.** Results of an animal model of ascending urinary tract infection.

503 A) HC14366 wild-type strain; B) HC14366M strain; C) HC14366MC strain.

504 Vertical axis, cfu/ml urine/gr. Bladder/ two kidneys

505

506 **Figure 2.** RT-PCR of the strains studied.

507 MC, HC14366MC strain; M, HC14366M strain; wt, HC14366 wild-type strain.

508

509 **Figure 3.** 2D-SDS page protein gels.

510 A) HC14366 wild-type strain; B) HC14366M strain; C) HC14366MC strain.

511

512 **Figure 4.** Real-time PCR of the genes selected.

513 HC14366, wild-type strain; HC14366M, mutant strain; HC14366MC, complemented

514 strain.

515

Table 1. Minimal Inhibitory Concentration (mg/L) of the strains under study.

Strain	CIP	NAL	NAL+Inh	NX	NX+Inh	C	C+Inh
HC14366wt	0.008	3	0.19	0.047	0.125	6	2
HC14366M	2.56	>256	>256	6	16	24	4
HC14366MC	0.064	6	0.38	0.5	0.5	16	3

HC14366wt, wild-type strain; HC14366M, *gyrA*-mutant strain; HC14366MC, complemented strain; CIP: ciprofloxacin; NAL, nalidixic acid; Inh, efflux pump inhibitor Phe-Arg- β -naphthylamide; NX, norfloxacin; C, chloramphenicol.

Table 2. Proteins characterized by 2D-SDS PAGE.

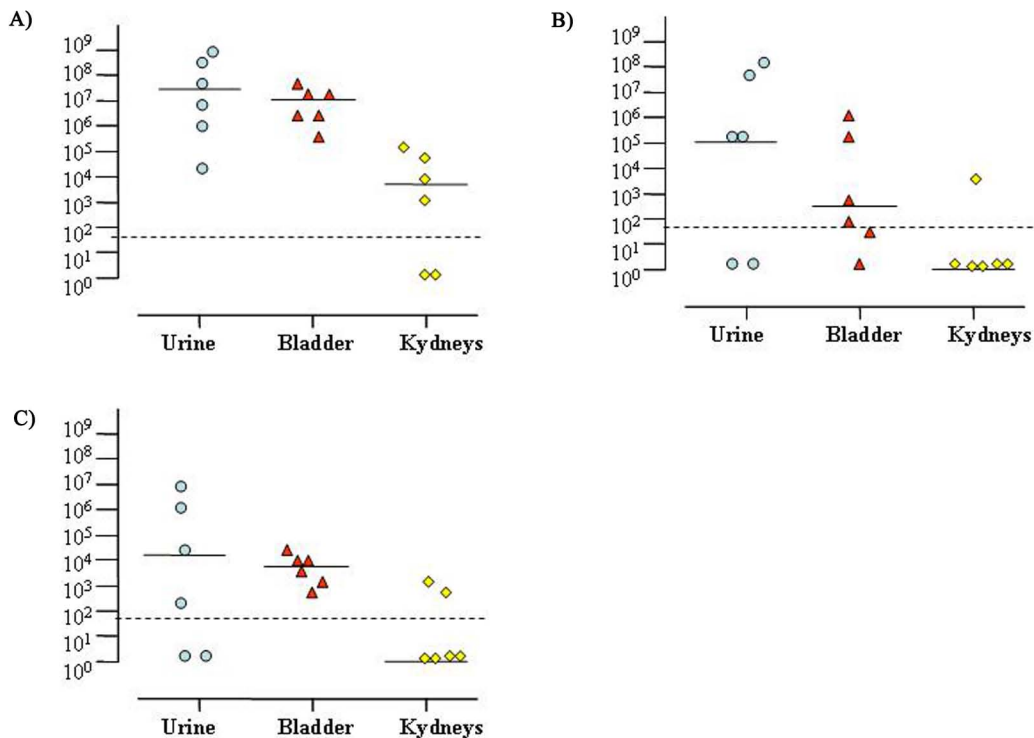
<i>ID number</i>	<i>Protein</i>	<i>Spot intensity</i>		
		HC14366wt	HC14366M	HC14366MC
0J	Aspartate ammonia-lyase	+++	+	+++
3J	Glycerol kinase	+	-	-
6J	Outer membrane protein (OmpF)	++	+	-
7J	Maltose-binding periplasmic protein precursor	+++	+	++
8J	Aminomethyltransferase	++	+	+
9J	Outer membrane protein A (OmpA)	++	-	++
11J	PTS enzyme IIAB, mannose specific	++	-	+
12J	D-ribose periplasmic binding protein	+++	+	++
13J	DNA-direct RNA polymerase	+	++	+
14J	Pyruvate kinase I	++	+	++
15J	6-phosphogluconate dehydrogenase	+	++	+
16J	Succinyl-CoA synthetase	+	++	++
18J	Duhydrolipoamide dehydrogenase	++	+++	++
20J	Tryptophanyl-tRNA synthetase	+	-	+
21J	Phosphate acetyltransferase	+	-	+
22J	HtpG, heat shock protein	-	+	-
24J	Adenylsuccinate synthetase	++	-	+
25J	Phosphoglycerate kinase	+++	+	++
26J	Tratrinat semialdehyde reductase	+	-	+
28J	Isocitrate dehydrogenase	-	+	-
29J	Cell division inhibitor	+	-	++

ID number, identification number from Figure 3.

HC14366wt, wild-type strain; HC14366M, *gyrA*-mutant strain; HC14366MC, complemented strain.

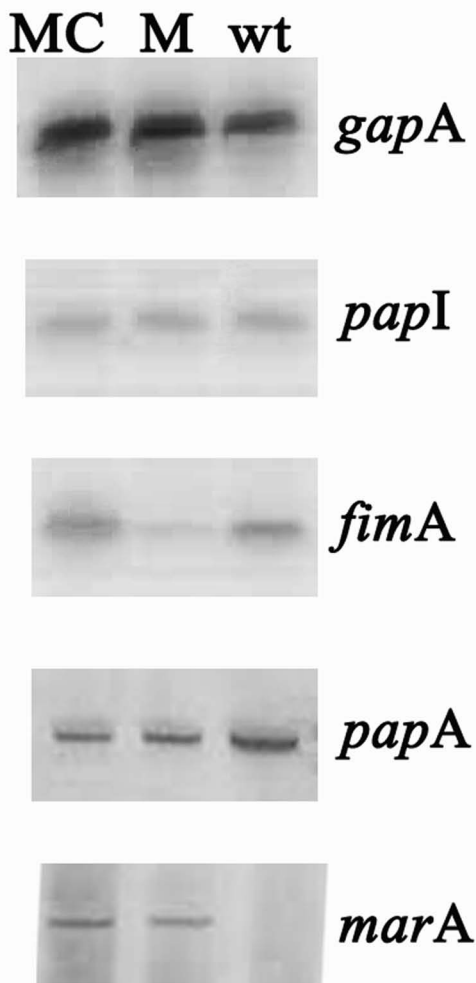
+++, high expression; ++, moderate expression; +, low expression; -, no protein expression.

Figure 1. Results of an animal model of ascending urinary tract infection.



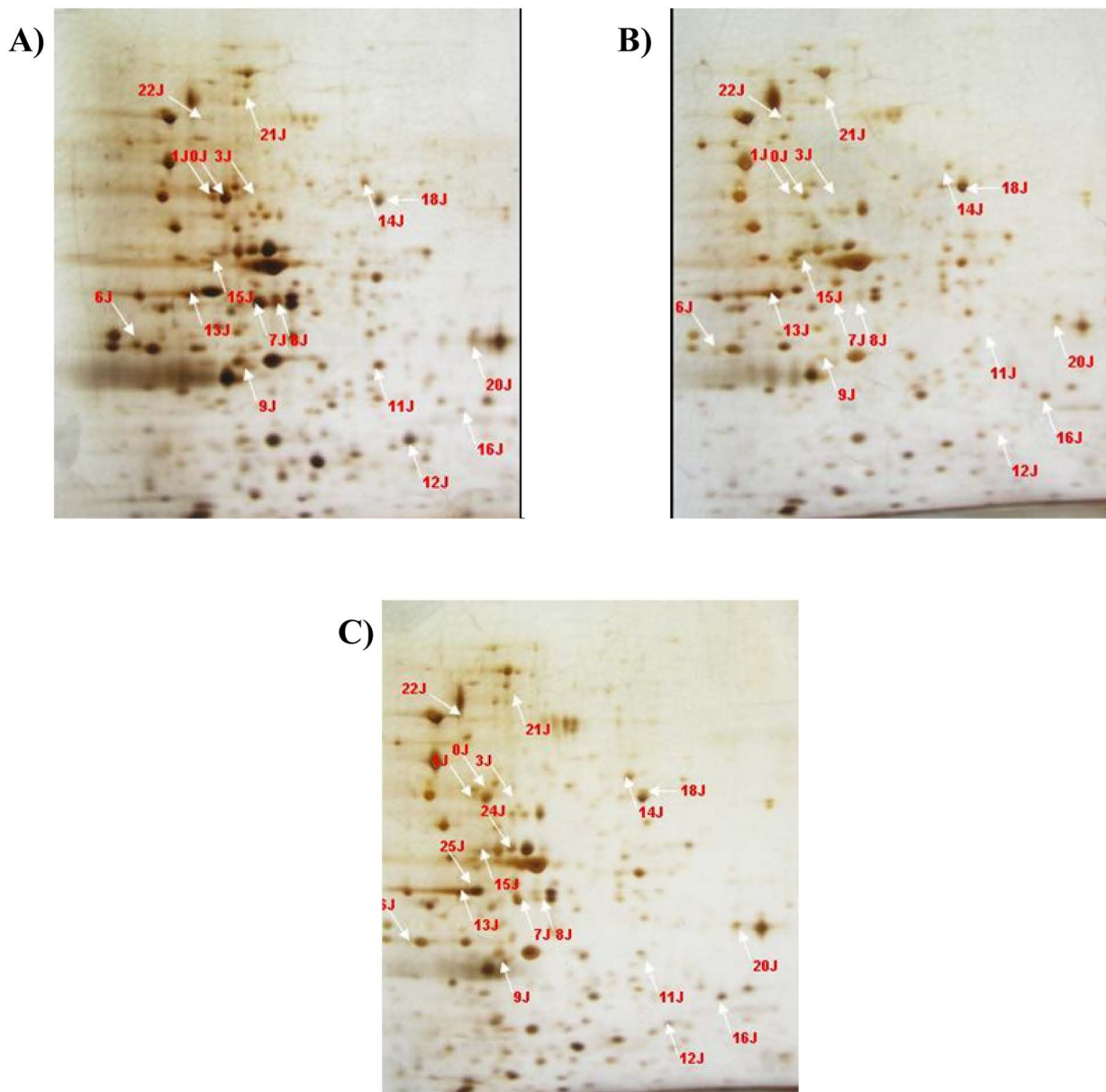
A) HC14366 wild-type strain; B) HC14366M strain; C) HC14366MC strain.
Vertical axis, cfu/ml urine/gr. Bladder/ two kidneys

Figure 2. RT-PCR of the strains studied.



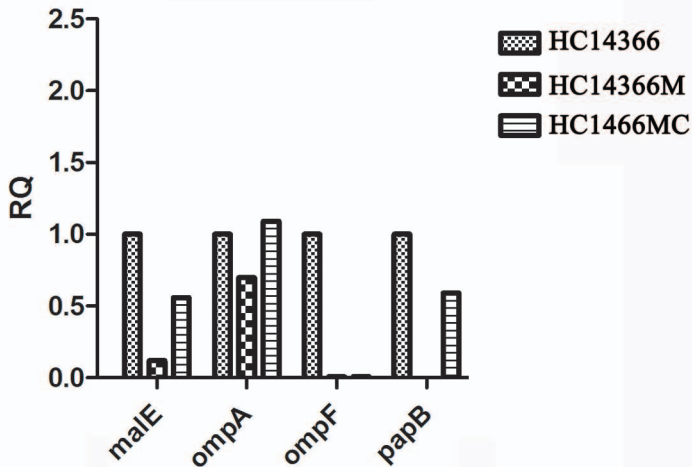
MC, HC14366MC strain; M, HC14366M strains;
wt, HC14366 wild-type strain.

Figure 3. 2D-SDS page protein gels.



A) HC14366 wild-type strain; **B)** HC14366M strain; **C)** HC14366MC strain.

Figure 4. Real-time PCR of the genes selected.



HC14366, wild-type strain; HC14366M, mutant strain;
HC14366MC, complemented strain.