TITLE: EFFECTS OF A MUTATION IN THE *gyr*A GENE ON THE VIRULENCE OF UROPATHOGENIC *Escherichia coli*

Running title: *gyrA* gene and virulence in UPEC

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

MATERIAL AND METHODS

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

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

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

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

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

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

Animal model. The virulence of the strains was tested in a murine model of an ascending UTI protocol approved by the Danish Ministry of Justice Animal Ethics Committee (approval no. 2004/561-835) and described by (22). In short, mouse bladders were emptied by gently pressing the abdomen, and 50 µl (5×10^6 CFU) of each bacterial suspension was slowly inoculated transurethrally into 4 to 6 outbreed female albino CFW-1 mice (26 to 30 g; Harlan Netherlands, Horst, Netherlands) with the use of plastic catheters. The mice were housed 4 to 6 to a cage and were given free access to food and 5% glucose-containing water. Seventy-two hours after inoculation, urine was collected from each mouse. The mice were then euthanized by cervical dislocation, and the bladder and kidneys were removed and stored in Eppendorf tubes. The urine samples were processed the same day by spotting (20 µl) of a series of 10-fold dilutions (10^0 to 10^{-6}) in duplicate on bromothymol blue agar plates (SSI Diagnostika, Hillerød, Denmark). The bladder and kidneys were stored in 0.9% saline 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 *fim*A gene
157 (GGACAGGTTCGTACCGCATC/ACGTTGGTATGACCCGCATC) generating an
158 amplicon of about 250 bp; the *mar*A gene (CATTCATAGCTTTTGGACTGGAT/GTG
159 TAAAAAGCGCGATTCGCC) generating an amplicon of about 150 bp; the *pap*A gene
160 (GGGGCAGGGTAAAGTAACTT/CAGGGTATTAGCATCACCT); and the *pap*I 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 (*fim*A, *mar*A, *pap*A and *pap*I
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

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

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

194 activation) followed by 40 cycles of 95 ºC for 15 s (denaturation) and 60 ºC
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

RESULTS

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

Tessier MC et al. (29) studied F165 adhesin from *E. coli*. This adhesin belongs to the family of Pap-related fimbriae the expression of which is mediated by regulatory proteins such as Lrp, Dam-methylase, and by FooI and FooB. They found that inactivation of the *gyrA* gene caused a decrease in supercoiling producing a decrease in *fooB* expression and inducing a decrease in P-fimbriae expression. FooB is the equivalent of PapB in the P-fimbriae. The decrease of *papB* expression found in the present study could explain the decrease of P-fimbriae expression, thereby making the mutant strain unable to adhere to renal epithelial cells and cause pyelonephritis. Although *papB* expression was recovered in the complemented strain, the finding that it did not recover the ability to cause pyelonephritis may be due to the fact that other P-fimbriae regulator (as PapI) were not affected by the inclusion of the plasmid containing the functional *gyrA* gene.

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

Treatment with fluoroquinolones can induce heat shock responses (30). For example, levofloxacin produced an overexpression of several heat shock proteins when the strain was incubated with this antibiotic (30), being HtpG one of these proteins. HtpG is the bacterial homologue of Hsp90 (presented in yeast and humans) and is dispensable under non-stress conditions. HtpG comprises a large fraction (0.36%) of all the proteins in *E. coli* growing at 37ºC (31). In the present study, this protein was found to be 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 10^2 CFU/g to 10^4 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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REFERENCES

1. **Calvo J, Martínez-Martínez L.** 2009. Antimicrobial mechanisms of action. Enferm Infecc Microbiol Clin **27:** 44-52.

2. **Hooper DC.** 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin Infect Dis **31** Suppl 2: S24-28.

3. **Martinez-Martinez L, Pascual A, Jacoby GA.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:** 797-799.

4. **Robicsek A, Jacoby GA, Hooper DC.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis **6:** 629-640.

5. **Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y.** 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother **51:** 3354-3360.

6**. Vila J, Ruiz J, Goñi P, De Anta MT.** 1996. Detection of mutations in the *parC* gene of quinolone-resistant isolates of *Escherichia coli*. Antimicrob Agents Chemother **40:** 491-493.

7. **Dorman CJ, Bhriain NN, Higgins CF.** 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. Nature **344:** 789-792.

8. **Vila J, Simon K, Ruiz J, Horcajada JP, Velasco M, Barranco M, Moreno A, Mensa J.** 2002. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? J Infect Dis **186:** 1039-1042.

9. **Horcajada JP), Soto S, Gajewski A, Smithson A, Jiménez de Anta MT, Mensa J, Vila J, Johnson JR.** 2005. Quinolone-resistant uropathogenic *Escherichia coli* strains from phylogenetic group B2 have fewer virulence factors than their susceptible counterparts. J Clin Microbiol **43:** 2962-2964.

10. **Takahashi A, Muratani T, Yasuda M, Takahashi S, Monden K, Ishikawa K, Kiyota H, Arakawa S, Matsumoto T, Shima H, Kurazono H, Yamamoto S.** 2009. Genetic profiles of fluoroquinolone-resistant *Escherichia coli* isolates obtained from patients with cystitis: phylogeny, virulence factors, PAIusp subtypes, and mutation patterns. J Clin Microbiol. **47:** 791-795.

11. **Soto SM, Jiménez de Anta MT, Vila J.** 2006. Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways. Antimicrob Agents Chemother **50:** 649-653.

12. **Kugelberg E, Löfmark S, Wretlind B, Andersson DI.** 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. J Antimicrob Chemother **55:** 22-30.

13. **Schilling JD, Mulvey MA, Hultgren SJ.** 2001 Structure and function of *Escherichia coli* type 1 pili: new insight into the pathogenesis of urinary tract infections. J Infect Dis **183** Suppl 1: S36-S40.

14. **Mulvey MA, Schilling JD, Hultgren SJ.** 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun **69:** 4572-4579.

15. **Plos K, Connell H, Jodal U, Marklund BI, Mårild S, Wettergren B, Svanborg C.** 1995. Intestinal carriage of P fimbriated *Escherichia coli* and the susceptibility to urinary tract infection in young children. J Infect Dis **171:** 625-631.

16. **Confer AW, Ayalew S.** 2013. The OmpA family of proteins: roles in bacterial pathogenesis and immunity. Vet Microbiol **163:** 207-222.

17. **Clinical and Laboratory Standards Institute (CSLI).** 2008. Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement M100-S17. USA.

18. **Ribera A, Doménech-Sánchez A, Ruiz J, Benedi VJ, Jimenez de Anta MT, Vila J.** 2002. Mutation in *gyr*A and *par*C QRDRs are not relevant for quinolone resistance in epidemiological unrelated *Stenotrophomonas maltophilia* clinical isolates. Microb Drug Resist **8:** 245-251.

19. **Johnson JR, Stell AL.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis**181:** 261-272.

20. **Andreu A, Fernandez F, Banus JM.** 1989. Type 1 fimbriae and P fimbriae in *Escherichia coli* producing chronic prostatitis. Enferm Infecc Microbiol Clin **7:** 131-134.

21. **Bagel S, Hüllen V, Wiedemann B, Heisig P.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob Agents Chemother **43:** 868-875.

22. **Hvidberg H, Struve C, Krogfelt KA, Christensen N, Rasmussen SN, Frimodt-Moller N.** 2000. Development of a long-term ascending urinary tract infection mouse model for antibiotic treatment studies. Antimicrob Agents Chemother **44:** 156-163.

23. **Martí S, Sanchez-Cespedes J, Oliveira E, Bellido E, Giralt E, Vila J.** 2006. Proteomic analysis of a fraction enriched in cell envelope proteins of *Acinetobacter baumannii*. Proteomics **6:** S82-87.

24. **Sorlozano A, Jimenez-Pacheco A, de Dios Luna Del Castillo J, Sampedro A, Martinez-Brocal A, Miranda-Casas C, Navarro-Marí JM, Gutiérrez-Fernández J.** 2014. Evolution of the resistance to antibiotics of bacteria involved in urinary tract infections: a 7-year surveillance study. Am J Infect Control. **42:** 1033-1038.

25. **Drlica K.** 1992. Control of bacterial DNA supercoiling. Mol Microbiol **6:** 425-433.

471 26. **Dove SL, Dorman CJ.** 1994. The site-specific recombination system regulating
472 expression of the type 1 fimbrial subunit gene of *Escherichia coli* is sensitive to changes
473 in DNA supercoiling. Mol Microbiol **14:** 975-988.
474
475 27. **Casadesús J, Low D.** 2006. Epigenetic gene regulation in the bacterial world.
476 Microbiol Mol Biol Rev **70:** 830-856.
477
478 28. **Hernday A, Krabbe M, Braaten B, Low D.** 2002. Self-perpetuating epigenetic pili
479 switches in bacteria. Proc Natl Acad Sci USA **99:** Suppl 4:16470-16476.
480
481 29. **Tessier MC, Graveline R, Crost C, Annick Desabrais, Martin C, Drolet M,**
482 **Harel J.** 2007. Effects of DNA supercoiling and topoisomerases on the expression of
483 genes coding for $F165_1$, a P-like fimbriae. FEMS Microbiol Lett **277:** 28-36.
484
485 30. **Yamaguchi Y, Tomoyasu T, Takaya A, Morioka M, Yamamoto T.** 2003. Effects
486 of disruption of heat shock genes on susceptibility of *Escherichia coli* to
487 fluoroquinolones. BMC Microbiol **3:** 16-23.
488
489 31. **Mason CA, Dünner J, Indra P, Colangelo T.** 1999. Heat-induced expression and
490 chemically induced expression of the *Escherichia coli* stress protein HtpG are affected
491 by the growth environment. App Environ Microbiol **65:** 3433-3440.
492
493 32. **Nicholson TF, Watts KM, Hunstad DA.** 2009. OmpA of uropathogenic
494 *Escherichia coli* promotes postinvasion pathogenesis of cystitis. Infect Immun **77:**
495 5245-5251.

33. **Graeme-Cook KA, May G, Bremer E, Higgins CF.** 1989. Osmotic regulation of porin expression: a role for DNA supercoiling. Mol Microbiol **3:** 1287-1294.

34. **Chubiz LM, Rao CV.** 2011. Role of the mar-sox-rob regulon in regulating outer membrane porin expression. J Bacteriol **193:** 2252-2260.

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 strain; 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.

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, *gyr*A-mutant strain; HC14366MC, complemented strain; CIP: ciprofloxacin; NAL, nalidixic acid; Inh, efflux pump inhibitor Phe-Arg-ß-naßhthylamide; NX, norfloxacin; C, chloramphenicol.

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

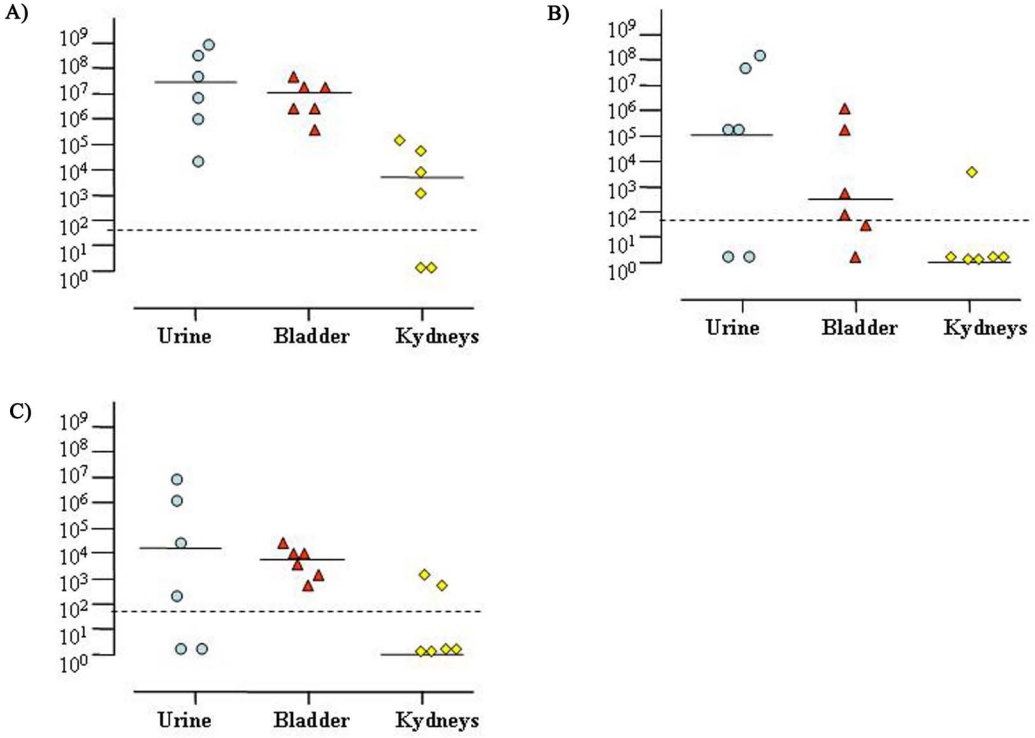
ID number	*Protein*	*Spot intensity*		
		HC14366wt	**HC14366M**	**HC14366MC**
0J	Aspartate ammonia-lyase	+++	+	+++
3J	Glycerol kinase	+	-	-
6J	Outer membrane protein (OmpF)	++	+	-
7J	Maltose-binding periplasmic protein precursor	+++	+	++
8J	Aminomethyltransferase	++	+	+
9J	Outer membrane protein A (OmpA)	++	-	++
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 sinthetase	+	++	++
18J	Duhydrolipoamide dehydrogenase	++	+++	++
20J	Tryptophanyl-tRNA synthetase	+	-	+
21J	Phosphate acetyltransferase	+	-	+
22J	HtpG, heat shock protein	-	+	-
24J	Adenylsuccinate synthetase	++	-	+
25J	Phosphoglycerate kinase	+++	+	++
26J	Tratrinate semialdehyde reductase	+	-	+
28J	Isocitrate dehydrogenase	-	+	-
29J	**Cell division inhibitor**	**+**	**-**	++

ID number, identification number from Figure 3.

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

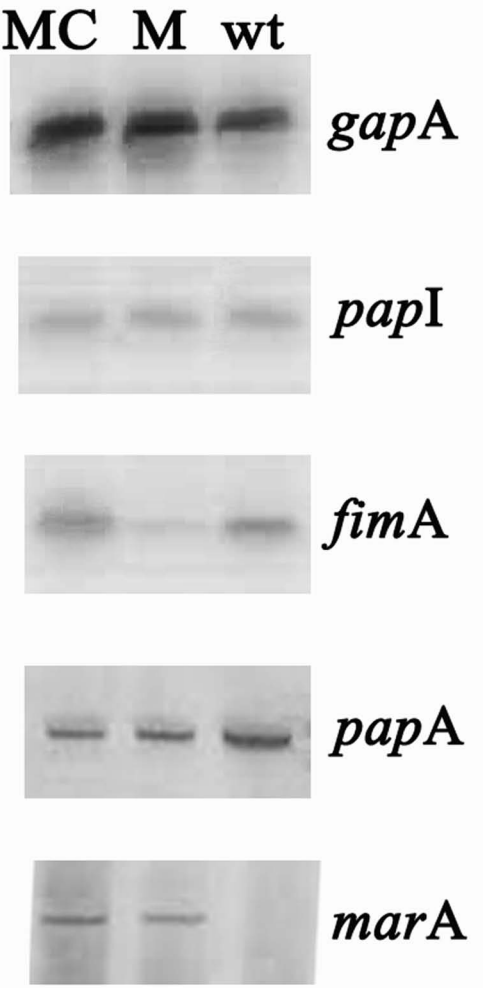
+++, high expression; ++, moderate expression; +, low expression; -, no protein expression.

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



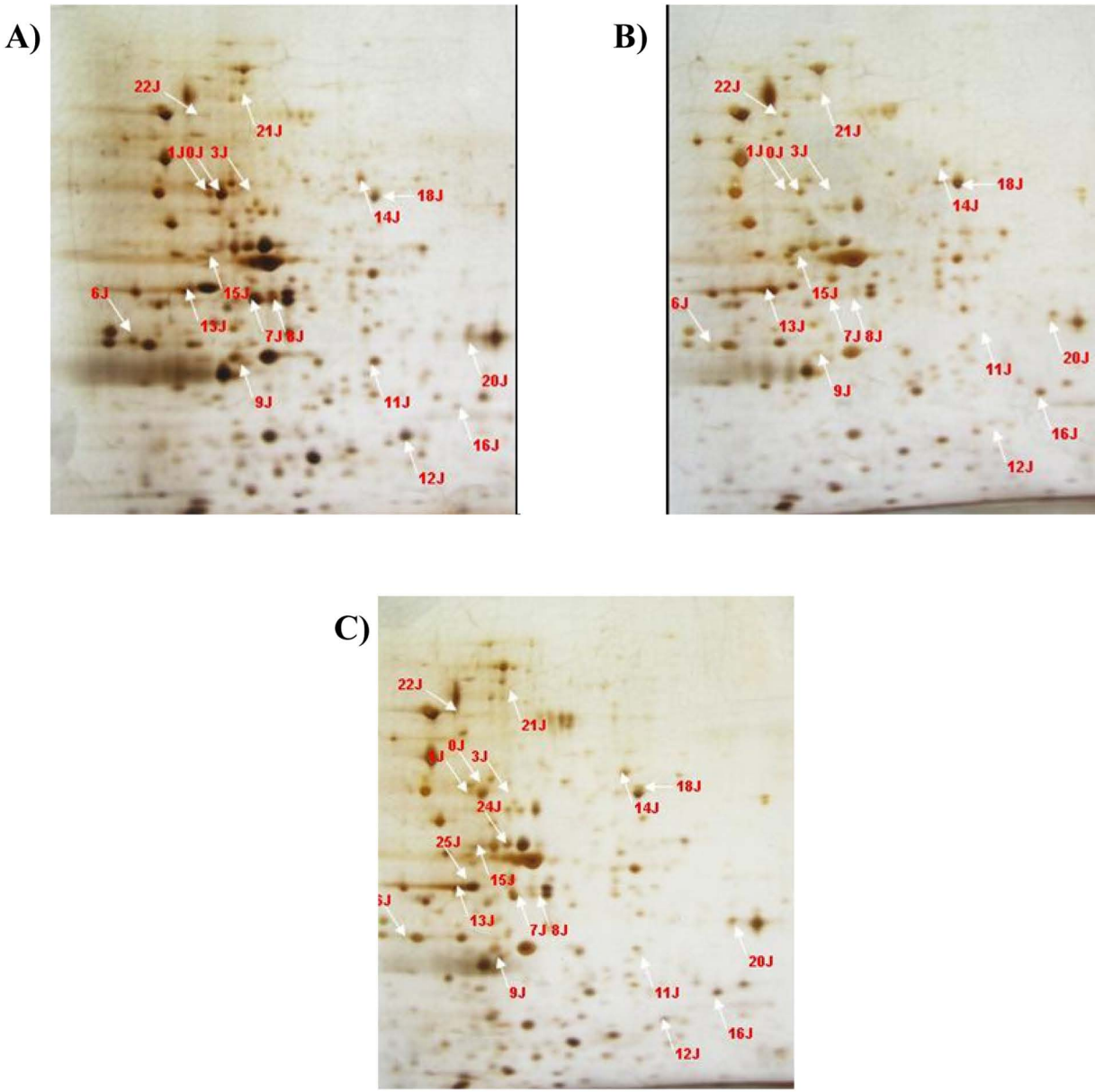
A) HC14366 wild-type strain; B) HC14366M strain; C) HC14366MC strain.
Vertical axis, cfu/ml urine/gr. Bladder/ two kidneys

Figure 2. RT-PCR of the strains studied.



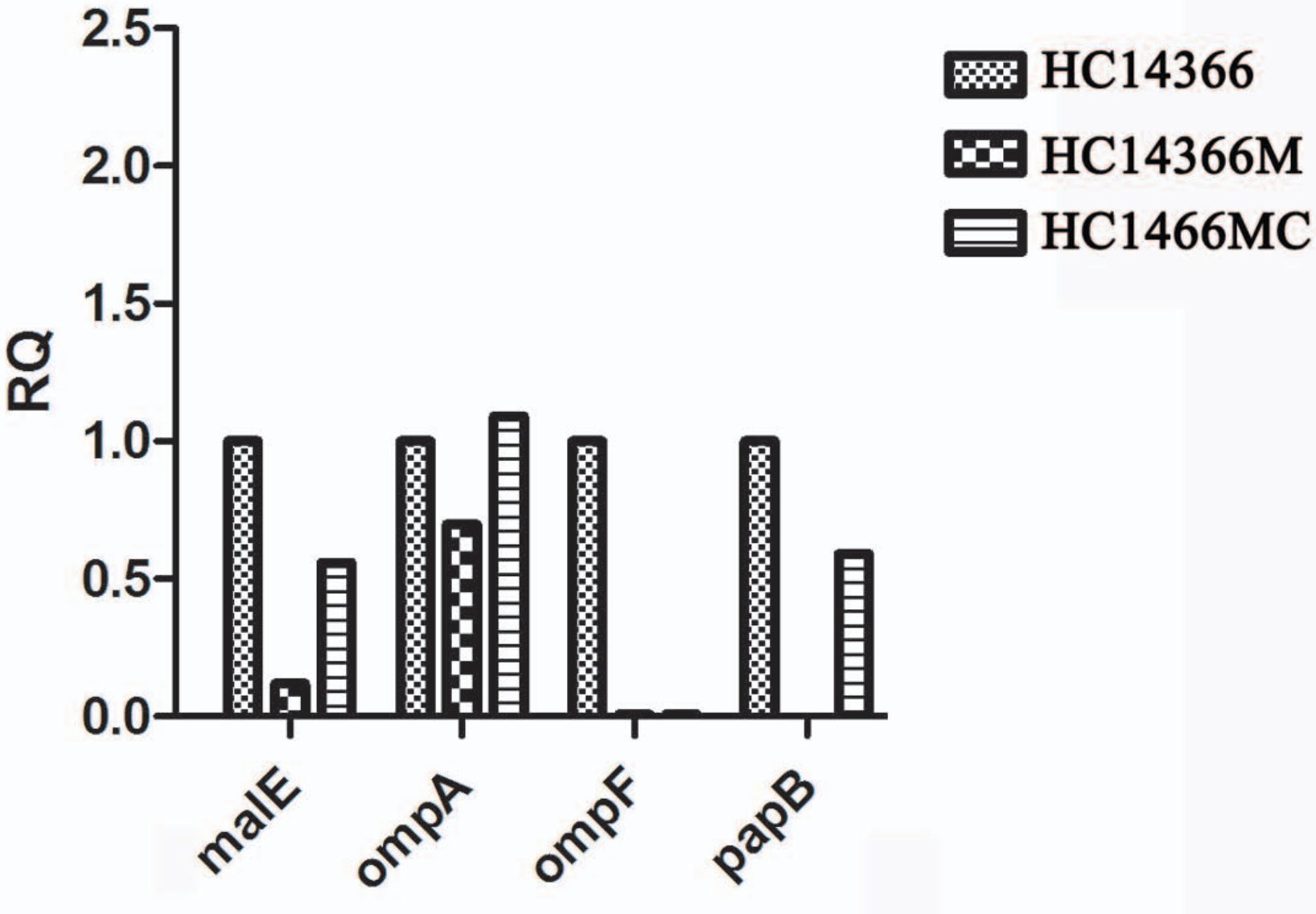
MC, HC14366MC strain; M, HC14366M strains; wt, HC14366 wild-type strain.

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.