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ORIGINAL ARTICLE

Adaptive strategies of uropathogenic *Escherichia coli* CFT073: from growth in lab media to virulence during host cell adhesion

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Abstract

Urinary tract infections (UTIs) are a major concern in public health. The prevalent uropathogenic bacterium in healthcare settings is *Escherichia coli*. The increasing rate of antibiotic-resistant strains demands studies to understand *E. coli* pathogenesis to drive the development of new therapeutic approaches. This study compared the gene expression profile of selected target genes in the prototype uropathogenic *E. coli* (UPEC) strain CFT073 grown in Luria Bertani (LB), artificial urine (AU), and during adhesion to host bladder cells by semi-quantitative real-time PCR (RT-PCR) assays. AU effectively supported the growth of strain CFT073 as well as other *E. coli* strains with different lifestyles, thereby confirming the appropriateness of this medium for in vitro models. Unexpectedly, gene expression of strain CFT073 in LB and AU was quite similar; conversely, during the adhesion assay, adhesins and porins were upregulated, while key global regulators were downregulated with respect to lab media. Interestingly, *fimH* and *papGII* genes were significantly expressed in all tested conditions. Taken together, these results provide for the first time insights of the metabolic and pathogenic profile of strain CFT073 during the essential phase of host cell adhesion.

Keywords Uropathogenic *Escherichia coli* · UPEC · Strain CFT073 · Gene expression profiles · Adherent bacteria

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Abbreviations

ABC-type	ATP-binding cassette
AU	Artificial urine
cAMP	3′-5′-Cyclic AMP
CFU	Colony-forming units
CRP	CAMP receptor protein
Ct	Cycle threshold
DAM	Deoxyadenosine methylase
ExPEC	Extra-intestinal pathogenic *E. coli*
FBS	Fetal bovine serum
Fnr	Fumarase-nitrate reductase regulator
GbO3	Globotriasylceramide receptor
GIs	Genomic islands
Hfq	Host factor Q-beta
HMDB	Human Metabolome Database
H-NS	Histone-like protein
LB	Luria Bertani
Lrp	Leucine-responsive regulatory protein
MOI	Multiplicity of infection
OD	Optical density
OMPs	Outer membrane proteins
PAIs	Pathogenicity islands
PBS	Phosphate-buffered saline

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PTS	Phosphotransferase system
RT-PCR	Real-time PCR
SD	Standard deviation
sRNA	Small RNA
UPEC	Uropathogenic *Escherichia coli*
UTIs	Urinary tract infections

Introduction

Urinary tract infections (UTIs) remain one of the most common infections of humans, associated with morbidity in both hospitalized as well as outpatients (Behzadi et al. 2021; Bruxvoort et al. 2020). Classified either as complicated and/or uncomplicated, UTIs can occur in any part of the urinary tract, causing urethritis, cystitis, and pyelonephritis, and progress to severe life-threatening urosepsis (Flores-Mireles et al. 2015; Spaulding and Hultgren 2016). UTIs are more common in women than man because of anatomical differences between sexes. Most uropathogens originate from the intestinal flora and ascend to the bladder via the urethra, with an interim phase of periurethral and distal urethral colonization, representing the primary site of infection in about 95% of all UTIs (Middendorf et al. 2001; Spaulding and Hultgren 2016). Uropathogenic *Escherichia coli* (UPEC) are the major causative agent of uncomplicated UTIs, responsible for up to 90% and 50% of community- and hospital-acquired infections, respectively (Behzadi 2020; Behzadi et al. 2021).

UPEC are included in the extra-intestinal pathogenic *E. coli* (ExPEC) group, which is characterized by a high degree of genetic heterogeneity with a carriage of a wide range of different virulence factors. Among UPEC, genetic diversities are largely attributed to the presence of virulence/fitness genes on mobile genetic elements referred to as pathogenicity islands (PAIs) or genomic islands (GIs) (Beatson et al. 2015). This array of virulence and fitness factors enables UPEC to access, colonize, and persist within the bladder and the kidneys (Behzadi 2020; Behzadi et al. 2021). It has been shown that the most studied prototype UPEC strain contains unique stretches of DNA, absent in other *E. coli* strains. Indeed, strain CFT073 carries 13 PAIs randomly inserted within the chromosome, of 30–100 kb each, accounting for about 12% of the genome. In CFT073, PAIs carry genes encoding proteins that contribute to pathogenesis, since the loss of these regions resulted in attenuate virulence (Mobley 2016). These genes encompass an array of fitness and virulence factors including, but not limited to, iron acquisition systems (siderophores and heme receptors), fimbriae and other adhesins, flagella, toxins, and metabolism (Behzadi et al. 2021; Sarshar et al. 2020; Scribano et al. 2020). Altogether these diverse virulence and fitness determinants enable UPEC strains to gain entry, adhere, acquire essential nutrients, multiply in a hostile environment, cause tissue damage, and disseminate within the urinary tract. Studying bacterial pathophysiology is therefore critical to understand infectious disease mechanisms. UPEC fitness and virulence determinants have been evaluated in a variety of laboratory settings, including UTI mouse models, while bacterial gene expression upon adhesion to host cells remains largely understudied. To this aim, major virulence, fitness, and metabolic determinants that enable UPEC to successfully survive and colonize the host urinary tract were investigated in strain CFT073. To better understand virulence mechanisms involved in human UTIs, the expression levels of these genetic determinants during cell adhesion were compared with bacteria grown in Luria Bertani (LB) and artificial urine (AU) media.

Materials and methods

Strain, media, and cell line

The well-characterized uropathogenic *E. coli* O6:K2:H1 strain CFT073 was used as uropathotype in this study (a kind gift from U. Dobrindt). The MG1655 and the enteropathogenic *E. coli* E2348/69 strains were retrieved from our laboratory collection. Routinely, strain CFT073 was grown at 37 °C in LB agar or seeded onto MacConkey agar plates, as previously described (Scribano et al. 2020). The AU medium was purchased from LCTech GmbH (Germany) and contained distilled water, deionized water (90–99%), urea (≤ 2.5%), creatinine hydrochloride (< 0.5%), sodium chloride (< 1.0%), sodium phosphate dibasic (< 0.5%), potassium phosphate (< 0.5%), sodium sulfite (< 0.5%), and ammonium chloride (< 0.5%). The human bladder epithelial cell line 5637, HTB-9 (ATCC-LGC, Milan, Italy), was routinely cultured in T25 flasks at 37 °C in a humidified atmosphere with 5% CO_2 using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Milan, Italy).

In silico *selection of marker genes and primer design*

The complete sequence of the *E. coli* O6:K2:H1 CFT073 (NC_004431) genome was used to select and analyze target genes in this study. Primers were partially picked based on previously published data (Alteri and Mobley 2007; Baum et al. 2014; Camprubí-Font et al. 2019; Chattopadhyay et al. 2015; Engelsöy et al. 2019; Kurabayashi et al. 2016; Li et al. 2016; Scribano et al. 2020; Subashchandrabose et al. 2013). Primers not previously described were designed with Clone Manager 6, using as criteria the following: target region of 190–260 bp, primer length at least of 19/20 bp, and calculated annealing temperature of 60 °C (Table 1 and Fig. 1).

Table 1 Primers used for RT-PCR analyses

Function*	Target gene	Primer sequence (5′-3′)	Reference
Iron acquisition systems	*iroN*	(F) AAGTCAAAGCAGGGGTTGCCCG (R) GACGCCGACATTAAGACGCAG	Alteri and Mobley (2007); Paniagua-Contreras et al. (2017)
	irp2	(F) AAGGATTCGCTGTTACCGGAC (R) AACTCCTGATACAGGTGGC	Paniagua-Contreras et al. (2017)
	chuA	F) AGCGTGTTGAGATTGTTCGC (R) AAACCACTGCTTTGTCCTTCCTGC	Alteri and Mobley (2007)
	iutA	(F) AAAGAGCTGAAAGACGCACTGG (R) TGTCGGAACGTGAAGAGTTGAG	Engelsöy et al. (2019)
	fhuA	(F) ACGGCCAAAGCCAGAATAAC (R) TTACCGTAAAGCACGGAAACCG	Alteri and Mobley (2007)
	feoB	(F) GCACTCTTTGTGCATGGTATTC (R) TGGCAGCACGGTGTTAAT	Li et al. (2016)
	fyuA	(F) ATGCCTATGTGGGATGGAATG (R) CCAGTCATCGGTGGTGTATTT	Li et al. (2016)
Solute transport	*ompA*	F) CTGGTGCTAAACTGGGCTG (R) TTAACCTGGTAACCACCAAAAG	Camprubí-Font et al. (2019)
	ompC	(F) CTGAGCAGCCAGGTAGATGTT (R) CATGCAGCAGCGTGGTAA	Camprubí-Font et al. (2019)
	ompF	(F) CGGCGTTGCTACCTATCGTA (R) CTGCCAGGTAGATGTTGTTCG	Camprubí-Font et al. (2019)
Fitness	*pafP*	(F) GCACTTGGTGGTAAAGAG (R) CTTTAGTGCCGATAATGACTTG	Baum et al. (2014)
	yddA	(F) CCTTAATCGCTGGTTTGCAG (R) TGTCTTCAGCGATACGTTGG	Subashchandrabose et al. (2013)
	yddB	(F) TACGGGTACAGGGACAGGAG (R) CATTTTATGTGGCGCTGATG	Subashchandrabose et al. (2013)
	pqqL	(F) TTTTGGTCGCGATGTTAATG (R) ATTGCCATCACTTGTTGCAG	Subashchandrabose et al. (2013)
	manX	(F) GGGCCAAACGACTACATGGTTATT (R) ACCTGGGTGAGCAGTGTCTTACG	Scribano et al. (2020)
	pgi	(F) CTCTGGCGAGAAGATCAACC (R) TCACCGGAAATAATCGCTTC	Engelsöy et al. (2019)
	ppsA	(F) GCAAAACAGGCCGTACAAAT (R) CAGCGTATAACGCTCCATGA	Engelsöy et al. (2019)
	gadA	(F) CAGGCAAACCAACGGATAAAC (R) CATAGGGATCTCACGCAACTC	This study
	gadC	(F) TCGAAGACCTTCTTCCCTGA (R) CATTACCCGGAATGACCATC	Chattopadhyay et al. (2015)
	hdeD	(F) CCGGTATTATCCGGTTTCCT (R) CTTTCATTGAACGCTGACGA	Chattopadhyay et al. (2015)
Adhesion	*sfaD*	(F) CTCCGGAGAACTGGGTGCATCTTAC (R) CGGAGGAGTAATTACAAACCTGGCA	Paniagua-Contreras et al. (2017)
	tsh	(F) GTCAGGTCAGTAACGAGCAC (R) AGAGACGAGACTGTATTTGC	Paniagua-Contreras et al. (2017)
	papG (allele II)	(F) GGGATGAGCGGGCCTTTGAT (R) CGGGCCCCCAAGTAACTCG	Paniagua-Contreras et al. (2017)
	fimH	(F) GCTGTGATGTTTCTGCTCGT (R) AAAACGAGGCGGTATTGGTG	Paniagua-Contreras et al. (2017)
	fimA	(F) ACTCTGGCAATCGTTGTTCTGTCG (R)ATCAACAGAGCCTGCATCAACTGC	Paniagua-Contreras et al. (2017)
	yadN	(F) CCACTGTTAATGGCGGTGTA (R) TTTAGCCAGGCGAGAAGAAC	Li et al. (2016)
Motility	*fliC*	(F) ACAGCCTCTCGCTGATCACTCAAA (R) GCGCTGTTAATACGCAAGCCAGAA	Kurabayashi et al. (2016)

Table 1 (continued)

Function*	Target gene	Primer sequence (5′-3′)	Reference
	motA	(F) GCTTTCTAATCTGAACGGTTACGC (R) TCCAGCTCAATAAACGACGGAC	Kurabayashi et al. (2016)
	cheW	(F) TTTGCCGTGACGCTTTCAACC (R) CGCCATCTCTTCGCTGT	This study
Protectins	*kpsM*	(F) CCATCGATACGATCATTGCACG (R) ATTGCAAGGTAGTTCAGACTCA	Paniagua-Contreras et al. (2017)
	ompT	(F) ATCTAGCCGAAGAAGGAGGC (R) CCCGGGTCATAGTGTTCATC	Paniagua-Contreras et al. (2017)
Toxins	*hha*	(F) GTGAACCTGCTGCCGATATC (R) ATTTGTGGATAAAAATGACG	Paniagua-Contreras et al. (2017)
	vat	(F) TACCGTAACCAGCTCATCAACAG (R) CATACCCACCTGTTACCCAATGT	Paniagua-Contreras et al. (2017)
Transcriptional regulators	*fur*	(F) TGTGGTTAGTCAGGCGAATG (R) GACAACCATCACGTCAGTGC	This study
	zur	(F) TGAAGTATGGGTGGGCTGAT (R) AAAGACCACAACGCAGGAGT	This study
	hfq	(F) TCCGTTCCTGAACGCACT (R) ACCGGCGTTGTTACTGTGAT	This study
	fnr	(F) ACGCTGTTTAAGGCTGGAGA (R) CCTTTGATTTCACCGCTCAT	This study
	crp	(F) GGGTGATTTTATTGGCGAAC (R) GTCCGGGTGAGTCATAGCAT	This study
Internal control	*nusA*	(F) TGAAGCCGCACGTTATGAAG (R) TCAACGTAATTCGCCCAGGTT	Campilongo et al. (2014)

*The functional category of each gene analyzed is reported

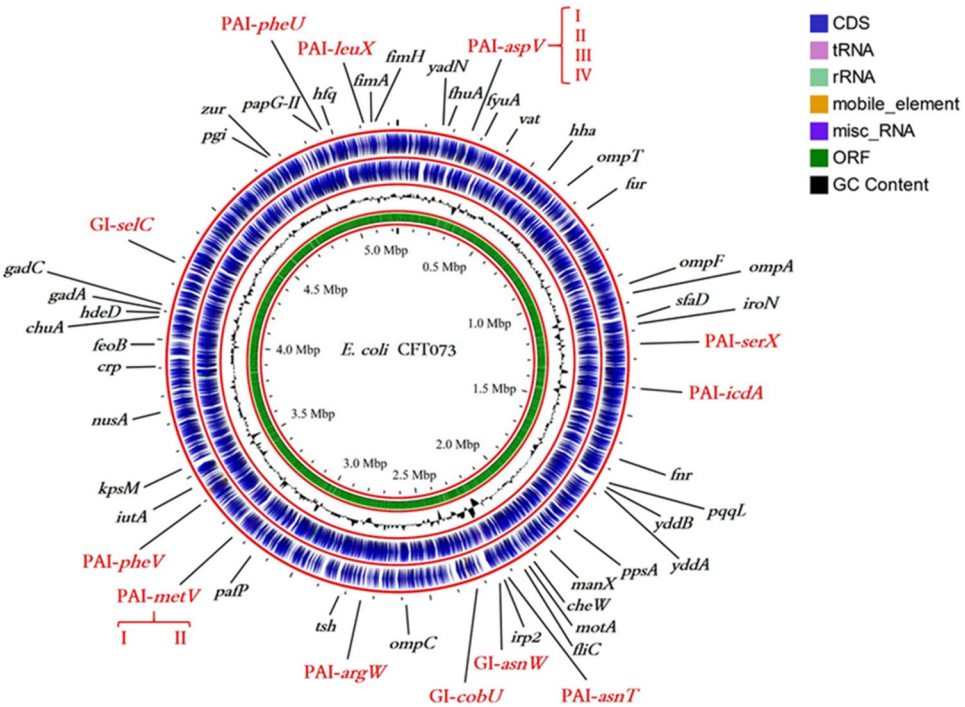

Fig. 1 Map of the *E. coli* CFT073 genome. The pathogenicity islands (PAI) are shown relative to the genomic sequence. The location of the genes targeted by RT-PCR is indicated. The image was generated using the CGView Server BETA at http://stothard.afns.ualberta.ca/cgview server/

Measurement of growth kinetics in vitro

Isolated colonies were used to inoculate LB broth. Bacteria from overnight cultures were collected by centrifugation at 15,000 × *g*, washed with sterile phosphate-buffered saline (PBS), and used to inoculate pre-warmed LB medium or AU.

Growth kinetics of strains CFT073, MG1655, and E2348/69 in either LB or AU were determined over time both by optical density (OD_{600}) measurements and by colony-forming units per mL (CFU/mL) counted by spot-plating serial dilutions on LB agar plates in triplicate.

Adhesion assays

Confluent cell monolayers of human HTB-9 cells were infected with strain CFT073 at a multiplicity of infection (MOI) of 10, centrifuged at 900 × *g*, and incubated in PBS at 37 °C with 5% CO_2 for 2 h. Cell monolayers were extensively washed to remove unbound bacteria, and then lysed with Triton X-100 0.1% in PBS followed by centrifugation at 15,000 × *g* to recover the maximal number of adherent bacteria (Scribano et al. 2020). To rule out the presence of intracellular bacteria, a gentamicin assay (200 µg/mL) was performed in parallel and incubating cell monolayers for further 30 min (Ambrosi et al. 2015). Invasion rate was negligible, accounting for 0.015% of the adhesion rates.

RNA extraction and semi-quantitative real-time PCR (RT-PCR)

Bacteria grown to mid-exponential phase in LB and AU were normalized to 3.5×10^8 CFU/mL (Fig. 2). Bacteria recovered post-adhesion were normalized by CFU/mL counting. Total RNA was extracted using the RiboPure™ Bacteria Kit (Ambion, Thermo Fisher, Milan, Italy). The quantity and quality of the purified RNA were assessed by measuring the absorbance at OD_{260} and using formaldehyde agarose gel electrophoresis, respectively. cDNA was generated using PrimeScript™ RT Reagent Kit with a random primer hexamer mix and 1 µg of total RNA, following the manufacturer's instructions (Takara Bio, Ancona, Italy). Semi-quantitative RT-PCR was performed in Stratagene MX3000P real-time PCR detection system using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent, Rome, Italy); the following conditions were used: 95 °C for 10 min, subsequently 40 cycles at 95 °C, 15 s, annealing at 60 °C, 30 s, and elongation at 72 °C, 30 s, followed by 5 min of final elongation. A temperature gradient from 58 to 70 °C for the annealing step was used in the temperature optimization assays. The sizes of RT-PCR products were checked by agarose gel electrophoresis. Each reaction was performed three times in triplicate. Cycle threshold (Ct) values achieved were normalized to the endogenous control, *nusA* (encoding glyceraldehyde 3-phosphate dehydrogenase), and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used for the comparative analyses. Results were reported as mean ± standard deviation (SD) and calculated as fold change of gene expression.

Statistical analyses

Unpaired Student's *t* test was used to determine statistical significance (GraphPad Prism 8.4.2). *P* values ≤ 0.05 were considered to be statistically significant.

Results and discussion

Artificial urine supports the growth of *E. coli* with different lifestyles

It has been reported that human urine can support the growth of uropathogens, including UPEC strains during urinary infections (Gond et al. 2018; Vejborg et al. 2012). Thus, to study the growth kinetics of the prototype UPEC strain CFT073 in physiological conditions most similar to human

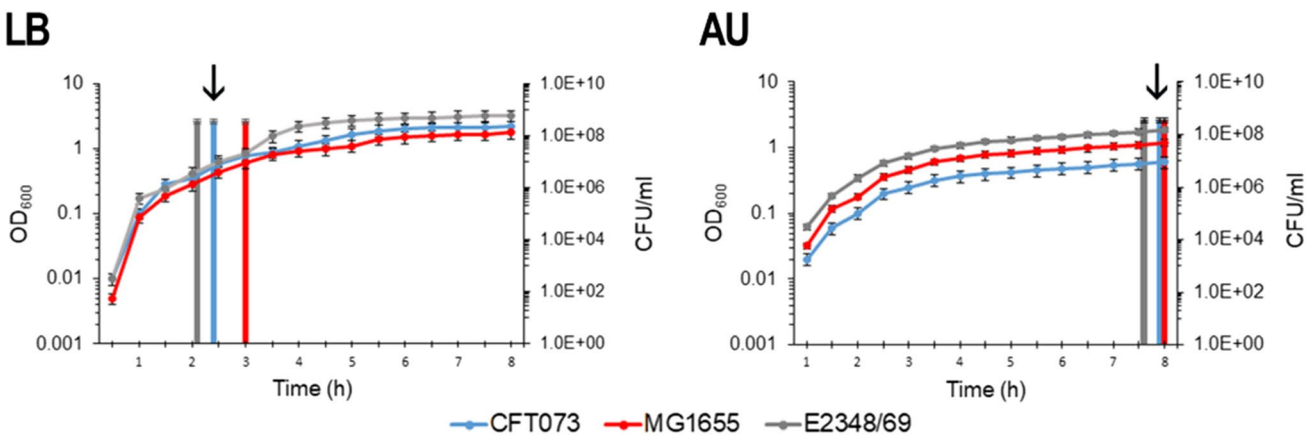

Fig. 2 Different *E. coli* strains are able to grow in AU. Growth kinetics of strains CFT073, MG1655, and E2348/69 in LB and in AU were monitored over time by measuring the OD at 600 nm. Bars indicate the time point in which each strain achieved an approximate number of 3.5×10^8 CFU/mL, measured by spot-plating appropriate dilutions. The arrows point to the bars corresponding to CFT073

urines, AU medium was used. As growth controls, a commensal and an enteropathogenic *E. coli* strains, MG1655 and E2348/69, respectively, were included. The growth kinetics in AU were determined by optical density measurements (OD_{600}) and by CFU counting, over time (Fig. 2). AU cultures under gentle shaking conditions at 37 °C reached OD_{600} values of 0.55 to 0.60 after 8 h, which corresponded to a mean value of 3.5×10^8 CFU/mL (± SD, of 1.7×10^7). These growth rates are in line with previously reported values for strain CFT073 in human urine (Ma et al. 2018). No significant differences were observed in the growth rates for strain CFT073 with respect to other *E. coli* strains (data not shown). These results show that the ability to grow in AU is not limited to strain CFT073, but it is shared among *E. coli* strains characterized by different lifestyles. However, this result does not explain the selective colonization of the urinary tract by UPEC strains (Alteri and Mobley 2007). In Gram-negative bacteria, the small and polar molecule urea can be produced intracellularly from purine catabolism and/or by the urea cycle, but it can also enter the cells passively or actively via ABC-type (ATP-binding cassette) transporters (Sachs et al. 2006; Sebbane et al. 2002). Apart from being used for buffering acidic conditions, urea can be broken down by urease, generating CO_2 and ammonia ($NH4^+$) which allow anabolic processes, such as the biosynthesis of amino acids and other cell components. Furthermore, it was recently demonstrated that growth of *E. coli* in urea was dose dependent (Taabodi et al. 2020). However, human urine is a high complex body fluid, containing also a number of different metabolites; at present, in pooled human urine, the Human Metabolome Database (HMDB) identified 115,398 metabolite entries in different concentrations, including amino acids, organic acids, and lipids (https://hmdb.ca/). Therefore, the extreme adaptability of UPEC strains allows them to survive by tolerating some metabolites and grow by catabolizing other ones (Reitzer and Zimmern 2019) (https://www.genome.jp/dbget-bin/www_bget?M00029).

Minor differences in gene expression profiles were shown by strain CFT073 grown in AU with respect to LB

To evaluate how strain CFT073 adapts its expression profile to AU in comparison with LB, specific target genes encoding for iron uptake, solute transport, protectins, toxins, motility, adhesion, and fitness, as well as global regulatory proteins, were selected (Fig. 1 and Table 1). Thus, total RNA was extracted from bacteria grown to mid-exponential phase in either LB or AU, retrotranscribed and analyzed by semi-quantitative RT-PCR using the primers reported in Table 1. Interestingly, only four genes showed significant differential gene expression in our experimental conditions, *fimA*, *fimH*, *papG,* and *crp* (Table 2). The expression of genes encoding proteins involved in CFT073 adherence, *fimA* and *fimH*, representing the major structural protein and the tip adhesin of the type 1 pili, respectively, was down- and upregulated in AU with respect to LB ($P < 0.01$ for both genes) (Table 2). Additionally, the expression of the *papG* gene encoding the tip adhesin of P pili was significantly upregulated ($P < 0.01$). The *fimA* and *fimH* genes, belonging to the *fimAICDFGH* operon, are frequently found in both UPEC as well as in human and animal intestinal *E. coli* strains (Ambrosi et al. 2019; Ma et al. 2018; Sarshar et al. 2017). Expression of type 1 pili is particularly important in UPEC strains since they mediate bacterial adhesion to host cells via the interaction of FimH with mannosylated uroplakins and α3,β1

Table 2 Semi-quantitative RT-PCR performed on RNA isolated from strain CFT073 grown in either LB or AU. The mean of log2 of relative quantity (RQ), calculated with the $2^{-\Delta\Delta Ct}$ method, ± standard deviation (SD) of each condition is shown. The data set was analyzed by unpaired Student's t tests. *P* values < 0.05 were considered statistically significant. Fold changes in bold represent significantly differentially expressed genes

AU vs. LB				
Target	**log2 (RQ_LB) ± SD**	**log2 (RQ_AU) ± SD**	**Fold change**	***P* value**
tsh	−2.50E-16 ± 2.25E-01	1.31E + 00 ± 1.53E + 00	2.48	2.16E-01
papG	6.29E-16 ± 6.22E-01	6.47E + 00 ± 2.85E-01	**88.54**	8.14E-05
fimA	−2.70E-01 ± 8.35E-01	−3.57E + 00 ± 2.27E-01	**0.10**	2.74E-03
fimH	6.52E-16 ± 3.00E-01	6.29E + 00 ± 1.12E-01	**78.43**	4.44E-06
pafP	0.00E + 00 ± 1.43E + 00	2.10E + 00 ± 5.06E-01	4.28	7.45E-02
manX	0.00E + 00 ± 7.70E-01	1.64E + 00 ± 8.22E-01	3.11	6.54E-02
vat	0.00E + 00 ± 6.46E-01	3.61E + 00 ± 2.19E + 00	12.17	5.23E-02
fur	−2.31E-17 ± 1.21E-01	3.74E + 00 ± 2.39E + 00	13.32	5.41E-02
zur	0.00E + 00 ± 4.05E-01	2.87E-01 ± 1.84E + 00	1.22	8.05E-01
hfq	−9.25E-17 ± 4.60E-01	4.93E-01 ± 3.07E + 00	1.41	7.97E-01
crp	0.00E + 00 ± 2.58E-01	−4.62E + 00 ± 6.02E-01	**0.04**	2.59E-04
fnr	0.00E + 00 ± 5.55E-01	1.25E + 00 ± 2.06E + 00	2.37	3.68E-01
ompA	0.00E + 00 ± 2.43E-01	4.53E-01 ± 5.38E-01	1.37	2.54E-01
ompC	−2.96E-16 ± 6.09E-01	6.03E-01 ± 1.30E-01	1.52	1.69E-01
ompF	0.00E + 00 ± 4.26E-01	−2.47E-01 ± 1.21E + 00	0.84	7.56E-01

integrins located on the bladder epithelium and underlying layers, respectively (Ribić et al. 2018; Sarshar et al. 2020; Scribano et al. 2020).The increased expression of *fimH* in AU is in agreement with a previous study performed in human urine (Subashchandrabose et al. 2014). Vice versa, the downregulated expression of *fimA* was quite unexpected. Hence, we reasoned that the rich LB medium allows the biosynthesis of FimA; in AU, conversely, CFT073 upregulates only *fimH* expression as an energy saving strategy in that it is metabolically less expensive, but it allows prompt piliation if the bacterium comes into contact with both abiotic and/or biotic surfaces. Nevertheless, we cannot rule out that *fim* expression profile could be due to differences in mRNA stability, ribosomal binding sites, and codon usage, as previously suggested for this operon (Klemm et al. 2010; Schembri et al. 2002). Another explanation could be linked to the control of cAMP receptor protein (CRP) (Müller et al. 2009). This transcriptional regulator modulates the expression of a number of bacterial metabolic and virulence genes by interacting with its co-factor cAMP, which is responsible for CRP conformational change (Shimada et al. 2011). Since cytoplasmic levels of cAMP are inversely correlated to glucose intracellular concentration, in glucose-depleted conditions, the complex CRP-cAMP can interact with specific DNA sequences on target genes, regulating their expression, a process known as carbon catabolite repression (Shimada et al. 2011). Both phase variation and *fimA* promoter activity are under negative control of CRP-cAMP; however, considering that some traces of glucose are found in human urine, the decrease in cAMP concentration could lead to an increased expression of type 1 pili (Müller et al. 2009; Scribano et al. 2020). However, in our experimental conditions, glucose or glucosides were absent in the AU medium. Therefore, it is reasonable to conclude that the composition of AU used in this study prevented *fimA* gene expression by inducing a phase OFF orientation of the promoter region *fimS* (Greene et al. 2015); vice versa, the growth in LB medium promoted the expression of type 1 pili. Intriguingly, some extent of *fimH* expression was reported even during phase OFF orientation of *fimS* in type 1 fimbrial phase-locked mutants of strain CFT073 (Snyder et al. 2005).

Additionally, the expression of the *papG* gene was significantly upregulated in AU. Expression of P pili is typical of UPEC strains responsible for upper UTIs (Behzadi 2020). The apical adhesin PapG is encoded by the *papAH-CDJKEFG* operon, regulated by two regulators, PapB and PapI, via reversible phase variation (Blomfield 2001). Three different PapG variants, known as PapGI, II, and III, were found among UPEC clinical isolates, differing in receptor binding properties (Behzadi 2020). Strain CFT073 carries two homologous *pap* gene clusters, P1 and P2, each encoding the *papGII* allele, which allows recognition of the globotriasylceramide (GbO3) receptor on uroepithelial cells in the kidneys (Behzadi 2020; Sarshar et al. 2020). It was previously reported that in strain CFT073 cultured in human urine, P pili were expressed mostly by the P1 gene cluster (Holden et al. 2007). Expression of the *pap* operon is controlled by a number of hierarchically organized regulators, such as H-NS, CRP, Lrp, DAM, PapI, the CpxAR signal transduction system, and the small regulatory RNA *papR* (Barnard et al. 2004; Werneburg and Thanassi 2018). Indeed, expression of P pili via phase variation is controlled by Lrp concentration, which depends on growth phases and conditions, being ON during the exponential growth phase (Werneburg and Thanassi 2018). Furthermore, a cross-talk regulates the expression of chaperone/usher pilus gene clusters via phase variation; in particular, P pili expression represses type 1 fimbrial expression, through the regulation of PapB on the *fimS* switch in strain CFT073 (Holden et al. 2007; Werneburg and Thanassi 2018). Overall, our results on pilus gene expression are in line with those achieved with *E. coli* 83972; a microarray approach with this *E. coli* strain allowed to find out that *pap* genes were significantly upregulated (Roos et al. 2006), whereas the *fimH* gene was upregulated in human urine (Subashchandrabose et al. 2014).

Interestingly, we found that expression of *crp* was significantly downregulated in AU with respect to LB (Table 2). Transcription of *crp* is autoregulated and depends on the concentrations of CRP and cAMP (Hanamura and Aiba 1991). In the absence of CRP-cAMP, RNA polymerase can transcribe the *crp* gene from its functional promoter; vice versa, in the presence of CRP-cAMP, it binds to a CRP site downstream its promoter, thereby allowing transcription of a divergent gene, thus inhibiting its own transcription (Hanamura and Aiba 1991). Therefore, in a glucose-depleted medium as AU, the levels of CRP-cAMP should be enough to induce a downregulation of the *crp* gene with respect to LB.

Iron is a critical nutrient for most bacteria, being a fundamental prosthetic component of a number of key enzymes involved in vital biological processes (Semsey et al. 2006). It has been reported that the content of iron in *E. coli* ranges between 10^5 and 10^6 atoms per cell, but iron is not bioavailable in mammals because it is sequestered in heme and nonheme proteins (Semsey et al. 2006); therefore, commensal and pathogenic *E. coli* developed multiple systems to acquire this precious metal from host cells during the course of infection (Ellermann and Arthur 2017). Strain CFT073 has fourteen outer membrane receptors for iron uptake, three siderophore biosynthetic systems, and several TonB-dependent receptors involved in iron transport (Snyder et al. 2004). Although reported before in human urine, no signal could be detected for iron uptake genes in either AU or LB (Snyder et al. 2004). While LB is rich enough in metals to repress iron uptake genes (Finney and O'Halloran 2003), we believe that iron content in AU was not low enough to derepress

iron uptake genes; either some external iron contaminants or some iron traces might have been present in AU in sufficient amounts to sustain strain CFT073 iron demand and keep iron uptake genes switched off.

Furthermore, a slight increase in the expression profile of the outer membrane proteins (OMPs) *ompA* and *ompC* was detected in AU with respect to LB (Table 2), although not statistically significant ($P > 0.05$); vice versa, an opposite trend was observed for *ompF*. However, these results are in good agreement with the differential expression of OMPs shown by strain CFT073 cultured in human urine and analyzed by a proteome approach (Alteri and Mobley 2007). Despite an increase in the expression of *ompW*, *ompC*, and *ompF*, no statistical significance could be assigned to these genes in that study; conversely, *ompA* showed a modest, statistical significant increase in human urine (Alteri and Mobley 2007). As highlighted by Alteri and Mobley, there is a wide variability in the expression profile of OMPs among different UPEC strains depending on the medium used for bacterial growth (Alteri and Mobley 2007).

Finally, no statistically significant results were achieved for *pafP*, *manX*, *vat*, *fur*, *zur*, *hfq*, and *fnr* and no signal for the other genes tested (Tables 1 and 2).

Genes encoding adhesins and outer membrane proteins are mainly involved in adhesion to bladder cells

Adhesion to host cells represents a critical step for UPEC in that allows them to contact, colonize, and eventually establish the infection. Thus, we wanted to compare the gene expression profile of strain CFT073 during cell adhesion with respect to the growth in LB and AU. For this purpose, an adherence assay was performed using the bladder cell line HTB-9. The RT-PCR analysis showed the same statistically significant increase in the expression of *papG* and *fimH* in adherent bacteria as in LB and AU (Tables 2 and 3). These data demonstrate that expression of both pili starts in AU and is maintained during cell host adhesion.

During cell adhesion, a high level of expression was also achieved for genes encoding bacterial OMPs, *ompA*, *ompC*, and *ompF* with respect to LB and AU growth conditions (Tables 2 and 3). Accordingly, a similar expression profile of OMP genes was reported for strain CFT073 upon adhesion to confluent monolayers of the human bladder cell line UM-UC-3 (Hagan and Mobley 2007). Indeed, OMPs of Gram-negative bacteria are well known to play several roles in nutrient uptake as well as interaction and persistence within bladder cells (Ambrosi et al. 2012; Scribano et al. 2016). In addition, it has been reported that OMPs contribute to UPEC survival under environmental stresses by contributing to structural support (Wang 2002). The expression of *ompA* in vivo was found to reach even higher levels when compared to urine levels (Hagan and Mobley 2007; Snyder et al. 2004). However, the expression of *ompA* was also found to be downregulated in vivo in a murine model with respect to bacteria grown in LB, whereas this expression profile was not reported for a chicken model (Zhao et al. 2009). Several conclusions can be drawn; (i) OmpA expression is host dependent; (ii) the balance between its crucial

Table 3 Semi-quantitative RT-PCR performed on RNA isolated from strain CFT073 during host cell adhesion (ADH) and from bacteria grown in either LB or AU. The mean of log2 of relative quantity (RQ), calculated with the $2^{-\Delta\Delta Ct}$ method, ± standard deviation (SD) of each condition is shown. The data set was analyzed by unpaired Student's *t* tests. *P* values < 0.05 were considered statistically significant. Fold changes in bold represent significantly differentially expressed genes

Target	ADH vs. LB				ADH vs. AU			
	log2 (RQ_LB) ± SD	**log2 (RQ_ADH) ± SD**	**Fold change**	***P* value**	**log2 (RQ_AU) ± SD**	**log2 (RQ_ADH) ± SD**	**Fold change**	***P* value**
tsh	−2.50E-16 ± 2.25E-01	−4.26E + 00 ± 1.61E + 00	**0.052**	1.06E-02	0.00E + 00 ± 1.53E + 00	−5.57E + 00 ± 1.61E + 00	**0.021**	1.23E-02
papG	6.29E-16 ± 6.22E-01	7.53E + 00 ± 4.23E-01	**184.823**	6.50E-05	5.92E-16 ± 2.85E-01	1.06E + 00 ± 4.23E-01	**2.087**	2.26E-02
fimA	−2.70E-01 ± 8.35E-01	−9.13E-01 ± 1.57E + 00	0.640	5.66E-01	5.90E-01 ± 4.40E-01	3.39E + 00 ± 1,57E + 00	**6.972**	4.12E-02
fimH	6.52E-16 ± 3.00E-01	7.12E + 00 ± 4.58E-01	**138.941**	2.31E-05	−1.08E-01 ± 1.12E-01	7.17E-01 ± 4.58E-01	**1.772**	3.88E-02
pafP	0.00E + 00 ± 1.43E + 00	−3.86E + 00 ± 6.41E-01	**0.069**	1.30E-02	1.85E-16 ± 5.06E-01	−5.96E + 00 ± 6.41E-01	**0.016**	2.26E-04
manX	0.00E + 00 ± 7.70E-01	−4.90E + 00 ± 7.94E-02	**0.033**	3.94E-04	8.33E-17 ± 8.22E-01	−6.54E + 00 ± 7.94E-02	**0.011**	1.63E-04
vat	0.00E + 00 ± 6.46E-01	−7.30E-01 ± 6.11E + 00	0.603	8.47E-01	0.00E + 00 ± 2.19E + 00	−4.34E + 00 ± 6.11E + 00	0.050	3.12E-01
fur	−2.31E-17 ± 1.21E-01	−4.67E-02 ± 4.06E + 00	0.968	9.85E-01	−8.85E-01 ± 2.39E + 00	−4.67E + 00 ± 4.06E + 00	0.073	2.37E-01
zur	0.00E + 00 ± 4.05E-01	−4.28E + 00 ± 2.24E + 00	**0.051**	3.10E-02	0.00E + 00 ± 1.84E + 00	−4.57E + 00 ± 2.24E + 00	0.042	5.22E-02
hfq	−9.25E-17 ± 4.60E-01	−8.45E + 00 ± 1.93E + 00	**0.003**	1.79E-03	−6.63E-01 ± 3.07E + 00	−9.60E + 00 ± 1.93E + 00	**0.002**	1.30E-02
crp	0.00E + 00 ± 2.58E-01	−9.20E + 00 ± 2.77E + 00	**0.002**	4.58E-03	0.00E + 00 ± 6.02E-01	−4.59E + 00 ± 2.77E + 00	**0.042**	4.86E-02
fnr	0.00E + 00 ± 5.55E-01	2.71E + 00 ± 4.63E + 00	6.536	3.72E-01	−6.42E-01 ± 2.06E + 00	8.20E-01 ± 4.63E + 00	2.754	6.44E-01
ompA	0.00E + 00 ± 2.43E-01	5.29E + 00 ± 1.92E + 00	**39.034**	9.15E-03	3.93E-175.38E-01	4.83E + 00 ± 1.92E + 00	**28.509**	1.38E-02
ompC	−2.96E-16 ± 6.09E-01	5.57E + 00 ± 2.63E + 00	**47.395**	2.35E-02	−2.94E-16 ± 1.30E-01	4.96E + 00 ± 2.63E + 00	**31.197**	3.11E-02
ompF	0.00E + 00 ± 4.26E-01	3.98E + 00 ± 2.04E + 00	**15.743**	2.97E-02	3.33E-16 ± 1.21E + 00	4.22E + 00 ± 2.04E + 00	**18.679**	3.67E-02

role in promoting and establishing the infection counterbalances its detrimental effects for its high immunogenicity; (iii) after a first phase of overexpression, it is possible that *ompA* expression is downregulated to prevent a massive immune induction. Further studies are required to fully elucidate the in vivo role of OmpA during the infective process.

Although undetectable in both LB or AU, adherent bacteria showed strong signals in RT-PCR also for *sfaD*, *flu*, and *yadN* genes, encoding the minor F1C fimbrial subunit, aggregation factor antigen 43, and the hypothetical fimbrial like protein precursors, respectively (data not shown). The lack of RT-PCR amplification of these genes in AU is coherent with their partial or strong downregulation previously reported in human urine (Paniagua-Contreras et al. 2017; Roos et al. 2006). Similarly, *sfaD* and *flu* were shown to be significantly downregulated also in LB (Snyder et al. 2004). Indeed, expression of adhesins is crucial for the establishment of a successful infection, since *E. coli* would be discarded by the bladder hydrodynamics. Thus, it can be concluded that during in vitro adhesion, strain CFT073 rearranges the expression of fimbrial genes when contacting bladder host cells, as reported for the in vivo conditions (Paniagua-Contreras et al. 2017; Sintsova et al. 2019).

Furthermore, iron-related OMPs (e.g., *iroN*, *iutA*, *fhuA*, *fyuA*, *chuA*) as well as *irp2* and *feoB*, encoding the yersiniabactin peptide synthetase and the iron transporter, respectively, were expressed following adhesion to host cells in our experimental conditions (data not shown). Unfortunately, we could not give any extent of expression due to the lack of these signals in both LB and AU growth conditions. However, the same expression profile involving OMPs and iron uptake genes was previously reported for strain CFT073 grown under conditions that mimic the urinary tract (Hagan and Mobley 2007). Thus, it might be concluded that adherent bacteria face nutrient and iron starvation during adhesion to host cells.

Genes involved in unavailable sugars are downregulated during adhesion to bladder cells

A statistically significant decrease in the expression *pafP* and *manX* was detected in adherent bacteria in comparison with those grown in LB and AU (Table 3). *pafP* is part of an operon together with *pafR*, and while *pafP* encodes a putative phosphotransferase system (PTS) sugar permease, *pafR* encodes a transcriptional regulator of genes involved in metabolism, virulence, and biofilm formation (Baum et al. 2014; Guyer et al. 1998). Likewise, *manX*, located within the *manXYZ* operon, encodes the EIIAB cytoplasmic component of the mannose PTS system (Erni et al. 1987; Scribano et al. 2020). Expression of *pafP* is dependent on the expression of PafR, which was shown to be moderately expressed in lab media as well as in a different AU medium in strain CFT073 (Baum et al. 2014); therefore, our results in both LB and AU are in agreement with this study, although without statistical significance (Table 2). Vice versa, the significant downregulation of *pafP* in adherent bacteria ($P < 0.05$) is likely due to the absence of any sugars during the adhesion assay; in fact, expression of *pafR* and thus *pafP* was suggested to be under CRP-cAMP control (Baum et al. 2014). Accordingly to previous data, it can be concluded that *paf* operon is generally poorly expressed in vitro, whereas it plays a role in virulence in the host (Baum et al. 2014; Mahan et al. 1993). On the other hand, despite being able to transport glucose into the cells, the PTS system to which ManX belongs, EIIABCDMan, represents the main mannose transporter (Deutscher et al. 2006). As for other sugar transporters, *manX* transcription is under catabolite repression (Aidelberg et al. 2014; Scribano et al. 2020). However, expression of this gene is quite complex having several levels of control, including Hfq and two small RNAs, SgrS and DicF (Azam and Vanderpool 2018). The *manX* downregulation paralleled the ones showed by both *crp* and *hfq*; therefore, during adhesion, strain CFT073 inhibits the expression of the *manXY* operon. Conversely, strong RT-PCR signals were observed from adherent bacteria for the following metabolic genes: *yddA*, *yddB*, *pgi*, *ppsA*, *pqqL*, *gadA*, *gadC*, and *hdeD* (Table 1 and data not shown), although the relative levels of expression were undetermined being not detectable in both LB and AU growth conditions.

Genes encoding toxins are poorly expressed during adhesion to bladder cells

It is well known that UPEC bacteria have the capability to produce and secrete toxins such as the hemoglobin protease Vat (also referred to as vacuolating autotransporter toxin) and the hemolysin expression-modulating protein Hha or Set (Nichols et al. 2016; Nieto et al. 1991). A significant reduction in the expression of *tsh* ($P = 0.01$) was recorded in bacteria from the adhesion assay in comparison with both LB and AU growth conditions (Table 3). Interestingly, the *tsh* gene, encoding a serine protease autotransporter, was rarely found to be detectable in the first phases of infection, being involved in invasion of the bladder epithelium (Hagan et al. 2010; Pokharel et al. 2020). Expression of *vat* was not statistically significant in our experimental conditions (Tables 2 and 3), while the expression of *hha* was detectable only in bacteria following contact with bladder epithelial cells, in line with previously reported data upon cell contact (data not shown) (Paniagua-Contreras et al. 2017). It was reported that Vat contributes to CFT073 fitness during bacteremia in a mouse model, whereas it seems to have poor, if any, serine protease activity during in vivo infections (Subashchandrabose et al. 2013). The Hha protein is a modulator of the expression of the toxin α-hemolysin in *E.*

coli (Nieto et al. 2000). It works in complex with H-NS, in response to environmental stimuli; in high osmolarity conditions or low temperature, the complex Hha/H-NS represses the hemolysin operon, most probably altering DNA topology (Nieto et al. 2000). Despite the *hha* gene was undetectable in either LB or AU, to the best of our knowledge, this is the first evidence that strain CFT073 expresses this gene during bladder cell adhesion.

Genes related to motility, capsule, and iron uptake are expressed only during adhesion to bladder cells

Most of UPEC strains, including CFT073, are motile; however, the role of flagella in their pathogenesis has not been elucidated yet. No RT-PCR signals were recorded for *fliC*, *motA*, and *cheW*, encoding flagellin, and chemotaxis proteins both in LB and AU. Conversely, their expression was appreciable in bacteria upon cell adhesion. Published data outlined that neither flagella nor chemiotaxis were required to colonize the bladder and kidney in a mouse model (Lane et al. 2005; Snyder et al. 2004). However, it is believed that flagella increase UPEC fitness during colonization of the urinary tract. In addition, it is worth mentioning that the flagellum has been shown to mediate adhesion to host cells as well as abiotic surfaces in *E. coli* (Haiko and Westerlund-Wikström 2013). Therefore, as suggested before, it can be hypothesized that flagella and chemiotaxis might assist the initial adhesion to host cells (Lane et al. 2005), while, later on, all genes related to flagella and chemotaxis in strain CFT073 were shown to be downregulated in vivo in comparison to LB (Snyder et al. 2004).

The genes *kpsM*, encoding an integral membrane protein involved in the translocation of the polysialic capsule (Vimr et al. 1995), and *ompT*, encoding an integral membrane endopeptidase (Kukkonen and Korhonen 2004), were expressed by strain CFT073 only during the adhesion assay, whereas their relative RT-PCR signals were not detected both in LB and in AU. The *kpsM* gene is genetically linked with *kpsT* gene, encoding the ATPase that is necessary for export of the capsule (Whitfield and Roberts 1999). Thus, it is reasonable that their expression raises during cell adhesion to increase bacterial adhesion ability. Furthermore and in line with previous observations, expression of *ompT* was detected only during the adhesion assay. The encoded protein, OmpT, was shown to cleave between pairs of the basic amino acids lysine and arginine, thereby being highly active on antimicrobial peptides secreted by host cells (Varadarajan et al. 2008). Accordingly, it was reported that its expression was not detectable in vitro in both LB and human urine, unless strain CFT073 was cultured in osmotic and low-iron conditions (Hagan and Mobley 2007). Moreover, OmpT was shown to participate to adhesion to bladder epithelial cells (Hasan et al. 2015), a role that is shared across different *E. coli* pathovars (Torres et al. 2020). Indeed, almost 10% of tested UPEC strains expressed *ompT* during infection of vulvar epidermoid carcinoma cells, although at a longer time point than our (Paniagua-Contreras et al. 2017).

In vitro contact with bladder cells was inducive for the expression of all iron-uptake genes, e.g., *fyuA*, *iroN*, *iutA*, *irp2*, *chuA*, *feoB*, and *fhuA* (Table 1). These genes encode the yersiniabactin (*fyuA*), salmochelin (*iroN*), aerobactin siderophore systems (*iutA*), iron repressible protein (*irp2*), ferrous ion transporter (*feoB*), and hemin and ferrichrome receptors (*chuA*, *fhuA*) (Robinson et al. 2018). As outlined before, *E. coli* pursued multiple iron acquisition strategies to fulfill its iron demand (Semsey et al. 2006). The iron-depleted niche within hosts triggers the expression not only of iron uptake genes, but also a plethora of virulence factors to successfully establish the infection (Kurabayashi et al. 2016). No statistically significant RT-PCR signal could be detected for *fur*, encoding the ferric uptake regulator, in all the analyzed conditions. Moreover, the global regulator Zur controls zinc uptake mechanisms as well as α-hemolysin, which effectively contributes to uroepithelial colonization (Patzer and Hantke 2000; Velasco et al. 2018). Expression of *zur* showed an opposite trend in cultured LB and AU vs. adherent bacteria; its downregulation was statistically significant only comparing data from the adhesion assay to LB growth conditions (Table 3). These results indicate that genes involved in host interaction are simultaneously derepressed to allow bacterial colonization, in line with previous published papers (Banerjee et al. 2020; Kurabayashi et al. 2016; Robinson et al. 2018).

Variable expression of key bacterial regulators during adhesion to bladder cells

Expression of *crp* was significantly downregulated in adherent bacteria in comparison with those grown in both LB and AU (Tables 2 and 3). The control exerted on gene expression by CRP-cAMP is not restricted to catabolite repression, but it is a global regulator and co-regulator of a huge number of genes in *E. coli* (e.g., 197), involved in general metabolism, host cell adhesion, and invasion (Gosset et al. 2004; Martínez-Antonio and Collado-Vides 2003). Downregulation of *crp* in adherent bacteria fits well with the increased adhesion to host cells for enhanced expression of *fim*, *kpsM*, and *pgi* genes and decreased expression of *pafP* and *manX* profile of CRP-regulated genes, which could represent the mark for the transition from urine to bladder epithelial cells. Additionally, CRP-cAMP regulates also the expression of the *hfq* gene encoding the RNA chaperone Hfq (Lin et al. 2011). This peculiar protein acts as an RNA-interacting factor, but it is also able to bind cellular DNA (Cech et al. 2016). In adherent bacteria, its expression was significantly downregulated in comparison with bacteria grown in LB and AU and

paralleled the expression profile shown by *crp* (Tables 2 and 3). In view of these results, it can be speculated that the host cell environment causes a downregulation of *hfq*, which, in turn, influences the stability of several target RNAs, such as *fur*, *gadAC*, and *manX* (Bianchi et al. 2020; Lin et al. 2011). Indeed, this interconnected regulatory cascade composed by CRP-cAMP/Hfq coordinates metabolic pathways, membrane homeostasis, motility, envelope stress, and virulence during UPEC adhesion steps and bacterial intracellular lifestyle later on (Behzadi et al. 2021).

Finally, no statistically significant differences among the three conditions tested were observed for the global regulator fumarase-nitrate reductase regulator (Fnr), encoded by the *fnr* gene (Tables 2 and 3). It regulates gene expression during adaptation to anaerobic growth conditions (Barbieri et al. 2014). Despite its proven role in the complex network governing UPEC pathogenesis and virulence together with the other key transcriptional regulators, it seems that Fnr plays an important gene expression control only when bacteria face hypoxic and anoxic conditions within the bladder (Marteyn et al. 2010).

Conclusions

UTIs caused by UPEC affect millions of people worldwide (Behzadi et al. 2021; Flores-Mireles et al. 2015; Foxman 2014). UPEC strains are different from commensal *E. coli* strains inhabiting the gastrointestinal tract due to the presence of additional genes encoding urovirulence factors that enable them to colonize and infect hosts (Mahan et al. 1993; Robinson et al. 2018). However, at the same time, strains belonging to the UPEC pathovar displayed an impressive heterogeneity, both in terms of genotypic and phenotypic characteristics that influence antibiotic and immune resistance, iron scavenging systems, additional metabolic enzymes, and structural components (Beatson et al. 2015; Guyer et al. 1998; Lloyd et al. 2009; Middendorf et al. 2001; Sarshar et al. 2017; Sintsova et al. 2019; Subashchandrabose et al. 2013). Despite being aware of these peculiar features, a core set of virulence factors common to all UPEC isolates has yet to be identified. Experiments performed in human urines added additional complexity to UPEC behavior; indeed, human urines were shown to contain a large number of different metabolites to which UPEC strains respond accordingly to their specific genomic content. Therefore, evaluating gene expression of different UPEC strains in a standardized defined medium could help to define if these bacteria use a common strategy to establish UTIs. In this study, we tried to give a picture of CFT073 gene expression switch during the first phases of host cell adhesion. The upregulation of adhesins and porins and the downregulation of global regulators with respect to lab media showed the deep gene expression reset of CFT073 during the contact with host cells. Apart from Fnr, regulons governed by CRP, Hfq, Fur, and Zur encode several virulence genes (Banerjee et al. 2020; Bianchi et al. 2020; Patzer and Hantke 2000; Shimada et al. 2011). The interplay among these global regulators that leads to the complex gene regulation cascades in response to environmental stimuli is still unknown and requires further studies.

Author information

This study was jointly conducted by authors working at the San Raffaele and Sapienza Universities.

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Declarations

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