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Genome-wide analysis of fitness factors in uropathogenic *Escherichia coli* in a pig urinary tract infection model

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) is the primary cause of urinary tract infections (UTIs) in animals and humans. We applied Transposon-Directed Insertion Site sequencing (TraDIS) to determine the fitness genes in two well-characterized UPEC strains, UTI89 and CFT073, in order to identify fitness factors during UTI in a pig model. This novel animal model better reflects the course of UTI in humans than the commonly used mouse model, and facilitates the differentiation between sessile and planktonic UPEC populations. A total of 854 and 483 genes in UTI89 and CFT073, respectively, were predicted to contribute to growth in pig urine, and 1257 and 764, were scored as required for colonization of the bladder. The combined list of fitness genes for growth in urine and cystitis contained 741 (UTI89) and 439 (CFT073) genes. The essential genes for growth on LB agar media supplemented with kanamycin and the fitness factors during growth in human urine were also analyzed in CFT073. A total of 457 essential genes were identified and the pool of fitness genes for growth in human urine included 215 genes. The gene *rfaG*, which is involved in lipopolysaccharide biosynthesis, was included in all the fitness-gene-lists and was further confirmed to be relevant for all the conditions tested regardless of the host and the strain. Thus, this gene may represent a promising target for the development of new therapeutic strategies against UTI UPEC-associated. Besides this important observation, the study revealed strain-specific differences in gene-essentiality as well as in the fitness-gene-repertoire for growth in human urine and UTI of the pig model, and it identified novel factors required for UPEC-induced UTIs.

1. Introduction

Urinary tract infection (UTI) is one of the most frequently diagnosed bacterial infections in humans. It is often associated with morbidity at hospitals and in the community, and up to 40% of adult women experience symptoms of cystitis during their lifetime (Nicolle, 2002; Andersen et al., 2022). Uropathogenic *Escherichia coli* (UPEC) is the predominant etiological agent in uncomplicated, community acquired UTIs (more than 50% of cases) (Wiles et al., 2008). The gut represents the natural habitat of UPEC, from where the pathogen can spread to the urinary tract and cause UTI. The transition from the gut to the bladder requires the expression of an arsenal of virulence factors as well as metabolic adaptation (Flores-Mireles et al., 2015; Terlizzi et al., 2017).

Broad-spectrum antimicrobials, including those classified as last-line antimicrobials, are frequently used to treat UTIs, and antibiotic resistance in UPEC is now widespread posing a threat to human health (Raeispour and Ranjbar, 2018). A recent report concluded that *E. coli* is the most important pathogen responsible for deaths attributable to bacterial antimicrobial resistance (Collaborators, 2022). To overcome the problem of antimicrobial resistance, it is crucial to identify novel targets for therapies to treat UTIs caused by resistant UPEC strains (for ex. phage-based therapies) as well as new targets for prevention strategies (for ex. vaccines) (Mann et al., 2017; Mazzariol et al., 2017). To do so, it is important to identify virulence and fitness factors, which are shared across UPEC strains and that are important for causing disease in different hosts (García et al., 2021). Lipopolysaccharide (LPS) synthesis

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represents one such fitness determinant, which has been demonstrated to be required during different types of UTI (growth in urine, cystitis and urosepsis) in several UPEC strains regardless of the host (Hagan et al., 2010; Phan et al., 2013; Aguiniga et al., 2016; Shea et al., 2020). In particular, the gene *rfaG* has been recently proved to be required for growth in urine, mouse cystitis and serum survival in different UPEC strains (Phan et al., 2013; Aguiniga et al., 2016; García et al., 2021).

In the present work, we apply Transposon-Directed Insertion Site sequencing (TraDIS), a gold standard high-throughput screening method to predict essential and fitness genes under specific test conditions (Langridge et al., 2009; Barquist et al., 2013). We assess Tn*5*-libraries constructed in two well-characterized UPEC strains; UTI89 (Mulvey et al., 2001) and CFT073 (Mobley et al., 1990), during infection of a recently developed porcine model for UTI - a model that recapitulates important aspects of human UTI - (Nielsen et al., 2019). Lists of fitness genes relevant for *in vivo* growth in pig urine as well as for colonization of the pig bladder were obtained. For comparisons with previous studies, the fitness-gene-set required for growth of CFT073 during growth in human urine and its essential-gene-set for growth on LB agar plates supplemented with kanamycin were also determined. While previous TraDIS-related studies focused on the analysis of genes contributing to fitness in one specific UPEC strain (Bahrani-Mougeot et al., 2002; Vejborg et al., 2012; Phan et al., 2013; Buckles et al., 2015; Shea et al., 2020), here, we compare the fitness landscape of the two strains during infection of the pig UTI model. The workflow followed in this study is depicted in Fig. 1.

2. Material and methods

2.1. Bacterial strains, genome comparison, generation of mutants and culture conditions

Strains used in this work are listed in Table 1. *E. coli* wild-type (WT) strains CFT073 and UTI89 were originally recovered from the blood and urine of a woman with acute pyelonephritis, and, from a patient with acute cystitis, respectively (Mobley et al., 1990; Mulvey et al., 2001). The genomes of 5.18 Mb (UTI89) and 5.23 Mb (CFT073), which are annotated and publicly accessible (Mobley et al., 1990; Mulvey et al., 2001; Welch et al., 2002; Chen et al., 2006; Luo et al., 2009), were compared using BLASTN (McGinnis and Madden, 2004) and the EcoCyc database (Keseler et al., 2017), to determine the genetic identity and detect common and differentially encoded proteins between the two strains.

Table 1
Strains used in this work.

Strain	Main features	Reference
UTI89	*E.coli* wild-type, clinical isolate from cystitis patient	(Mulvey et al., 2001)
CFT073	*E.coli* wild-type, clinical isolate from pyelonephritis patient	(Mobley et al., 1990)
CFT073-pKD46	UTI89 harboring the plasmid pKD46 with λ red recombinase expressed from an arabinose inducible promoter (Gen^R)	This work
UTI89 Rif^R	*E.coli* UTI89 (Rif^R)	(García et al., 2021)
CFT073 Rif^R	*E.coli* CFT073 (Rif^R)	This work
UTI89Δ*rfaG*	*E. coli* UTI89 lacking *rfaG* (Chl^R)	(García et al., 2021)
CFT073Δ*rfaG*	*E. coli* CFT073 lacking *rfaG* (Chl^R)	This work

Chl^R, Rif^R and Gen^R: chloramphenicol, rifampicin, and gentamicin resistant, respectively.

An isogenic strain of *E. coli* CFT073 resistant to rifampicin (CFT073 Rif^R) as well as a deletion mutant lacking the gene *rfaG* (CFT073Δ*rfaG)* were generated as described (Datsenko and Wanner, 2000; Weinstein and Zaman, 2019; García et al., 2021). CFT073Δ*rfaG* was verified by PCR and the gene-specific deletion was confirmed as previously stated (García et al., 2021). The growth of CFT073Δ*rfaG* and CFT073 Rif^R was compared with the growth of the WT CFT073 in Luria broth (LB) (Sigma) and EZ MOPS (Teknova) media by estimating the OD_{600} values at 0, 2, 4, 6, 8 and 24 h post-inoculation. Growth curves for each strain were obtained.

Strains were grown at 37 °C on LB agar or in LB broth. Antibiotics (Sigma) were added when required at the following concentrations: gentamicin (Gen) 15 μg/mL, kanamycin (Kn) 50 μg/mL,

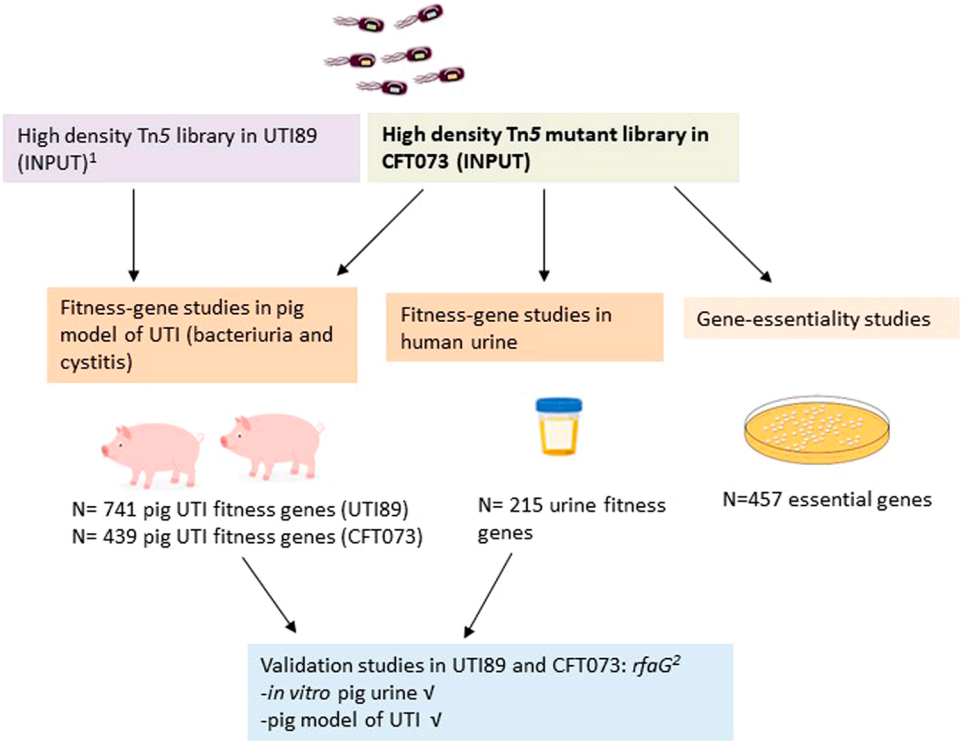

Fig. 1. Scheme of the workflow followed in this study. [1]The input Tn-libray in UTI89 was constructed in our previous work (García et al., 2021) where essential fitness genes for growth on LB agar plates supplemented with Kn, and fitness genes during *in vitro* growth in laboratory media and human urine as well as during mouse cystitis were identified. [2]The gene *rfaG*, also confirmed to be relevant during growth in human urine in CFT073 in the current study, was previously shown to play a role during growth in human urine and infection of the mouse model of UTI in UTI89 (García et al., 2021).

Table 2
TraDIS data-sets analysed in the present work.

Tn-Library (condition)	Total Reads[1]	Reads Mapped (%)[2]	Total UIS[3]	Total Seq Length/Total UIS
Input				
UTI89_1 (input 1)[4]	11,574,067	10,372,421 (89.6)	190,809	27.1
UTI89_2 (input 2)[4]	8331,970	8049,946 (96.6)	149,998	27.8
UTI89_1 +UTI89_2 (combined)[4,5]	19,906,037	18,422,370 (92.5)	222,483	23.3
CFT073_1 (input 1)	9396,997	8948,384 (95.2)	125,937	41.5
CFT073_2 (input 2)	8324,588	7930,213 (95.3)	121,608	43.0
CFT073_1 +CFT073_2 (combined)[5]	17,721,585	16,878,599 (95.2)	140,380	37.3
Output (urine)				
UTI89_pig_urineMIX_1 (2 pigs)[6]	6520,628	6030,996 (92.5)	105,071	49.3
UTI89_pig_urineMIX_2 (2 pigs)[6]	8761,911	8506,385 (97.1)	106,977	48.4
CFT073_pig_urineMIX_1 (2 pigs)[6]	6915,820	6710,583 (97.1)	128,325	48.8
CFT073_pig_urineMIX_2 (2 pigs)[6]	8343,607	8067,876 (96.7)	142,147	36.8
CFT073_human_urineT20_1	8627,985	8138,756 (94.3)	116,398	44.9
CFT073_human_urineT20_2	6735,112	6474,843 (96.1)	110,745	47.2
Output (bladdder)[6]				
UTI89_pig_bladder1_1 (pig 1)	8689,152	8482,348 (97.6)	55,600	93.2
UTI89_pig_bladder1_2	8582,108	8295,738 (96.7)	62,440	82.9
UTI89_pig_bladder2_1 (pig 2)	8638,224	8293,531 (96)	63,094	82.1
UTI89_pig_bladder2_2	9650,363	9317,798 (96.5)	64,399	80.4
UTI89_pig_bladderMIX1 (2 pigs)	8263,713	8037,906 (97.3)	74,118	69.9
UTI89_pig_bladderMIX2	8171,863	7935,892 (97.1)	81,614	63.4
CFT073_pig_bladder1_1 (pig 1)	9737,265	9410,546 (96.6)	91,580	57.1
CFT073_pig_bladder2_1 (pig 2)	8344,882	8130,937 (97.4)	88,356	59.2
CFT073_pig_bladder2_2	6050,666	5874,137 (97.1)	80,240	62.2
CFT073_pig_bladderMIX1 (2 pigs)	6266,834	6070,849 (97.9)	89,421	58.5
CFT073_pig_bladderMIX2	10,599,772	10,181,012 (96)	110.093	47.5

[1]Number of sequence reads with matching online barcode. [2]Number of mapped sequence reads against *E. coli* UTI89 or CFT073 genome (% of the raw data). [3]Number of Unique Insertions Sites (UIS). [4]These libraries have been previously obtained and analyzed (García et al., 2021). [5]Sequencing reads from duplicates of the input library were combined to maximize the coverage and analyze gene essentiality as previously described (García et al., 2021). [6]Output libraries derived from infection of the pig model of UTI analyzed in this work are: i) pig urine output libraries in duplicates: composite libraries representing the two pigs infected with the same library, and ii) bladder output libraries in duplicates: from individual pigs, and composite libraries representing the two pigs infected with the same library after pooling one mL of each of the two individual output libraries. Note that for CFT073_pig_bladder_1 (pig 1) there is only one replicate analyzed (CFT073_pig_bladder1_1)

chloramphenicol (Chl) 30 μg/mL or rifampicin (Rif) 50 μg/mL.

2.2. Construction of a high-density transposon mutant library (input library) in CFT073 and validation of random transposon insertion

Random Tn*5* transposon insertion mutants were obtained in *E. coli* CFT073 as described for UTI89 (García et al., 2021). Briefly, one μl EZ-Tn5™ <KAN-2 >Tnp Transposome™ (Epicentre) was electroporated into an aliquot of competent cells of CFT073. After electroporation and expression, cells were cultured overnight at 37 °C on large, Ø= 15 cm LB agar plates supplemented with Kn. CFUs were counted, and Kn resistant colonies from each plate were collected into sterilized LB supplemented with glycerol 20% (v/v). One mL aliquots of each expanded library of approximately 1×10^{10} CFUs were stored at − 80 °C. To verify that insertion of the transposon had occurred at random sites across the CFT073 genome, the PCR-based protocol "random amplification of transposon ends" (RATE) was applied (Ducey and Dyer, 2002). Approximately 1×10^{9} CFUs of the input expanded library were subjected to DNA extraction and sequencing for further analysis of the input pool (see below) (Table 2).

2.3. Testing of the CFT073 input library during growth in human urine

To facilitate comparison with previous studies (Shea et al., 2020; García et al., 2021), the input CFT073 Tn-library was tested during *in vitro* growth in human urine. In brief, freshly voided urine from two healthy women with no history of UTI or antibiotic use within the last two months was pooled and filter-sterilized. Filtered urine (9.9 mL) was inoculated with approximately 1×10^{9} viable Tn-mutants (0.1 mL) and further incubated at 37 °C for 20 h to obtain the output library "output CFT073_human_urine_T20″ (Table 2). Growth experiments were carried out in duplicate.

2.4. Pigs and anesthesia

Ten female pigs (Landrace/Yorkshire, mix) were purchased from a Danish supplier, Kokkenborg ApS, with the highest health status according to the Danish SPF system(https://spfsus.dk/en). The mean weight when the experiments began was 39.9 kg (SD: 4.2) and the age was between 13 and 14 weeks. Animals were kept in standard hay-covered pig enclosures provided with standard feed and *ad libitum* water.

For sedation, pigs were pre-medicated with intramuscular (i.m.) injection of medetomidine (Cepetor 0.12 $mg{\cdot}kg^{-1}$) (CP-Pharma, Germany), butorphanol (Butomidor 0.2 $mg{\cdot}kg^{-1}$) (Richter Pharma, Austria) and midazolam (midazolam 0.1 $mg{\cdot}kg^{-1}$) (Hameln, Germany). Anesthesia was induced and maintained on propofol (Orion Pharma, Finland) and fentanyl (B. Braun, Germany) after which the pigs were given i.m. atipamezol hydrochlorid (0.2 $mg{\cdot}kg^{-1}$) (Dechra, UK) to reverse the α2-adrenergic effects of medetomidine. The pigs were administered intravenous ringer-acetate (Fresenius Kabi, Sweden) at a rate of 1.4 $mL{\cdot}min^{-1}$ during sedation.

2.5. Infection of pigs with the UTI89 and CFT073 input Tn-libraries

Four sedated pigs were placed in a supine position and catheterized with a charrier 10 foley type catheter (Rüsch, Teleflex Medical, Ireland). At this time point, urine specimens were collected and cultured on LB agar plates to verify that no ongoing infection was present prior to the experimental inoculation. Urine specific gravity (USG) was also estimated using a refractometer (UG-α, Atago) (Andersen et al., 2012; García et al., 2021). Hereafter, the bladders were emptied, and a 100 mL suspension of each input Tn-library, (prepared directly from the glycerol stock containing approx. 1×10^{10} CFUs) in saline solution (0.9% NaCl), containing approx. 10^{8}-10^{9} CFUs, was immediately instilled into the bladder of two pigs. One hundred μl of each inoculum were ten-fold diluted and dilutions were plated on LB agar plates, which were incubated overnight at 37 ºC to enumerate Tn-mutants. At 3 h post-infection

(hpi), 100 mL of urine were removed from the bladders to avoid distention of the bladder. At 6 hpi, another 150 mL of urine were collected from each pig and aliquoted into 10 tubes with 10 mL each and one tube with 50 mL. All tubes were centrifuged at 200xg for 2 min to remove exfoliated bladder cells. The supernatants, containing the bacteria, were transferred to sterilized tubes and centrifuged at 5500xg for 20 min to pellet the bacterial cells. Pellets were stored at − 20 °C for further DNA extraction (Table 2). At this time point, the animals were euthanized and whole bladders were removed and transferred to a sterile laminar air-flow cabinet. Tissue-associated bacteria were quantified as described by Stærk et al. (2021). In short, the bladders were opened by a midline incision and 12 round-sized specimens (Ø=10 mm) were punched out from the bladder surface. The urothelium was extracted by peeling the epithelial layer from the underlying tissue using forceps and a scalpel and these specimens were homogenized in 10 mL saline solution. For bacterial quantification, 100 µl of each sample were ten-fold diluted and dilutions were plated on LB agar plates, which were incubated overnight at 37 ºC to determine CFU counts. The bladder output libraries were prepared as previously described (García et al., 2021). Briefly, the remaining 9.9 mL of each bladder suspension were plated onto Ø= 15 cm agar plates supplemented with Kn (approx. 0.5 mL suspension per plate) and incubated overnight at 37 ºC. Colonies were collected with LB supplemented with glycerol 20%, and four output libraries (one per bladder/pig) were obtained (Table 2). One mL from each of the generated output libraries was further mixed to obtain one composite output library representing the two pigs infected with the same library (Table 2). Aliquots of each expanded library were prepared as described and stored at − 80 °C (García et al., 2021).

2.6. Histology of bladders

Tissue sections of bladders from pigs inoculated with the CFT073 and UTI89 input Tn-libraries at 6 hpi were subjected to histological examinations. Bladder samples were collected and fixed in 10% (vol/vol) neutral buffered formalin phosphate (Fisher Scientific, Roskilde, Denmark), routinely processed for histology and paraffin-embedded. Sections were cut at 4 µm and stained with hematoxylin and eosin (H&E). The slides were examined with an Olympus BX45 light microscope with an attached DP25 digital camera (Olympus, Ballerup, Denmark). Histological grading was performed by a certified veterinary pathologist at the University of Copenhagen. A bladder sample from a healthy non-infected pig was used as a control.

2.7. DNA extraction, quality control and quantification. Preparation of the libraries for sequencing and Illumina sequencing

Genomic DNA was purified using the DNeasy blood and tissue kit (Qiagen) as recommended by the supplier from approx. 5×10^9 cells of the input and output Tn-libraries libraries (Table 2). Quality of DNA was confirmed by NanoDrop (Thermo Fisher), and DNA concentration was estimated by using Qubit® dsDNA HS Assay Kit (Thermo Fisher) (García et al., 2021).

Preparation of libraries for sequencing was performed essentially as described (Barquist et al., 2016; García et al., 2021). Briefly, the following steps were performed; approximately 2–3 µg of DNA were sheared into approx. 300 bp fragments using Covaris M220 (Covaris) followed by DNA end-repair, DNA end adenylation and adapter ligation (NEBNext® End Repair Module, NEBNext® dA-Tailing ModuleNEBNext® and Quick Ligation Module; New England Biolabs) using the adapter splinkerette design strategy (Barquist et al., 2016). This was followed by PCR enrichment of Tn-insertion fragments, and qualitative and quantitative verification through Bioanalyzer and qPCR. Sequencing was performed as reported (Barquist et al., 2016; García et al., 2021) using a MiSeq platform and a reagent kit V2 (50 cycles) (Illumina). Primers used for TraDIS have been described (García et al., 2021). The TraDIS sequence data from this study were deposited on the European Nucleotide Archive (ENA) under the study accession numbers PRJEB41961 and PRJEB52903.

2.8. Analysis of sequence data and statistics using the Bio::TraDIS pipeline

Analysis of sequencing data was performed essentially as described previously (Barquist et al., 2016; García et al., 2021) using the Bio::Tradis analysis pipeline (https://github.com/sanger-pathogens/Bio--Tradis). Briefly, the analysis allowed for estimation of the accurate insertion site of the transposon across the genome, and Unique Insertion Sites (UIS) and read counts were determined per gene. The read counts and UIS in CFT073 were visualized using Artemis version 17.01 (Carver et al., 2012), and circular genome diagrams were obtained by DNA-Plotter version 17.01 as previously performed with UTI89 (Carver et al., 2009; García et al., 2021).

Using the analysis script *tradis_essentiality.R*, gene essentiality was evaluated in the CFT073 input Tn-library. As a result, a bimodal distribution of insertion indexes (IIDs) (number of insertions per gene divided by gene length) for non-essential genes (gamma) and essential genes (exponential) was determined. Log_2 likelihood ratios (LLR) were calculated between the fitted distributions, and a gene was classified as essential if showing a LLR < -2, leading to an essentiality cutoff at an IID of 0.0008 for CFT073 in our data. A gene was classified as nonessential if showing a LLR > 2, giving an insertion index cutoff of 0.0014. IIDs falling between these two values were classified as "ambiguous".

Using the *tradis_comparisons.R* script, which establishes significant differences in read counts between input and output pools among non-essential genes, UPEC fitness genes during *in vitro* growth in human urine (CFT073), *in vivo* growth in pig urine and pig cystitis (CFT073 and UTI89) were predicted. Genes with a Q value of < 0.001 and an absolute log_2 fold change difference (logFC) of < -2 were considered significant. As previously proposed, a gene with a cut-off value LogFC > 2 and Q < 0.001 was considered an anti-fitness gene (García et al., 2021).

Predicted fitness genes common to all lists of genes obtained in UTI89 (mice bladder colonization, human bacteriuria (García et al., 2021), and UTI of pigs) were re-annotated using the UniProt database (Consortium, 2019).

CFT073 essential genes for growth on LB agar plates supplemented with Kn which showed zero or few Tn- insertions, and fitness genes predicted during growth in human urine, were functionally annotated using the EggNOG database (Huerta-Cepas et al., 2017, 2019) that assigns a Cluster of Orthologous Groups (COG) to each gene. They were plotted as fractions of the total number of genes in each functional category in the reference CFT073 genome. Fitness genes identified during pig UTI (common to growth in pig urine and bladder colonization) in both UPEC strains were also functionally annotated.

2.9. Assessment of the role of the gene rfaG during in vitro growth in pig and human urine

WT UTI89 and CFT073 and their corresponding mutants; UTI89Δ*rfaG* (García et al., 2021) and CFT073Δ*rfaG* (this study) were compared for the ability to grow in pig urine as previously described (García et al., 2021). Briefly, overnight cultures of each WT and the derived mutant strain lacking *rfaG* were adjusted to an OD_{600} of 0.05 in 10 mL of pig urine and incubated with shaking (180 rpm) at 37 °C. The OD_{600} was determined every hour from t = 0 h to t = 8 h, and at t = 24 h post-inoculation. A growth curve for each strain was obtained. For comparisons with previous studies, CFT073 Rif^R was also tested with CFT073Δ*rfaG* in competition assays in human urine as described (García et al., 2021) and growth ability was compared. In brief, overnight cultures of each strain were adjusted to an OD of 0.1, equally mixed and inoculated in 5 mL of urine. CFU counts of the inoculum were performed to confirm a ratio of 1:1. Suspensions were incubated at 37 °C with shaking for 24 h. Samples were collected every second hour from t = 0

to t = 8 h and then at t = 20 h and t = 24 h post-inoculation, and 10-fold dilutions were plated on LB agar plates supplemented with Rif (for detection of CFT073 Rif^R) or Chl (for detection of CFT073Δ*rfaG*). Plates were incubated overnight at 37 ºC, CFU counts were performed, and growth curves were generated. The experiments were performed at least in triplicates.

2.10. Assessment of the role of the gene rfaG during UTI in the pig model

Competition challenge experiments were carried out to confirm the role of the identified fitness gene *rfaG* during UTI in the pig model. For these experiments, pigs were acclimatized for 7 days before infection. Procedures were similar to those described above for testing of Tn-libraries. Once the bladders were emptied, pigs were inoculated with a 100 mL bacterial suspension in saline solution of UTI89 Rif^R and UTI89Δ*rfaG* (three pigs) or CFT073 Rif^R and CFT073Δ*rfaG* (three pigs). The inoculum was prepared as described previously (García et al., 2021) by re-suspending a few colonies of WT-Rif^R and the corresponding mutant, directly collected from plates after overnight growth, in 5 mL of saline solution. The OD was adjusted to 0.4, which corresponds to approx. 5×10^8 CFU mL^{-1} and an equal volume of the strains were mixed. The inoculum was then serially diluted for a final concentration of approx. 10^3 CFU·mL^{-1} of the UTI89 Rif^R+UTI89Δ*rfaG* suspension, and 10^6 CFU·mL^{-1} of the CFT073 Rif^R+CFT073Δ*rfaG* suspension. Aliquots were 10-fold diluted and dilutions were spread on agar plates with Rif (for detection of UTI89 Rif^R or CFT073 Rif^R) or Chl (for detection of the mutants). Plates were incubated overnight at 37 ºC to enumerate the inoculated bacteria.

Blood samples were collected at baseline (just before infection) and at 3, 6 and 24 hpi to assess systemic inflammation by differential white blood cell counts using a hematology analyzer (element HT5, Mindray). At 3 hpi, a urine specimen (approx. 20 mL) was collected, and 150 mL were emptied from the bladder to decrease bladder pressure. Urine (approx. 20 mL) was collected again at 6 hpi and 24 hpi. Urine bacteriuria was quantified at the three-time points (3, 6 and 24 hpi) by plating ten-fold serial dilutions on LB agar supplemented with the appropriate antimicrobials. At 24 hpi pigs were euthanized and whole bladders and kidneys were removed and examined by gross pathology. Bladders were processed as described above with the exception that only seven punch-outs were made and homogenized in 5 mL saline solution. Specimens from the renal parenchyma (superior and inferior pole) were homogenized and quantified similarly. One part of the bisected kidneys was also sonicated for 5 min in 40 mL saline to assess potential bacteria present in the renal pelvis. For bacterial quantification, 100 μl samples were serial diluted and plated on LB agar plates supplemented with the appropriate antibiotic, and plates were incubated overnight at 37 ºC. From each plate, at least two colonies were subjected to matrix-assisted laser desorption ionization -time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Billerica, MA, United States) and verified to be *E. coli*.

The competitive infection index (CI) was calculated as the ratio between mutant CFU and WT CFU found in the pig urine divided by the ratio of mutant CFU and WT CFU in the inoculum multiplied by 100, as previously described (Herrero-Fresno et al., 2014).

2.11. Statistical analysis

Statistical significance was determined by one-sample t-test using GraphPad Prism (version 8.3.0) (GraphPad Software Inc.). A p-value (P) below 0.05 was considered significant.

2.12. Ethical statement

Healthy women volunteers participated as urine donors after providing informed written consent. Experiments with pigs were conducted according to the ARRIVE guidelines, the principles expressed in the Declaration of Helsinki, and were approved by the Danish Animal Experiments Inspectorate, license numbers: 2019–15–0201–01626 and 2021–15–0201–00931.

3. Results

3.1. General features of the UPEC genomes and verification of the CFT073 input library

Comparative genomics using BLASTN and EcoCyc databases revealed 99.02% identity with 92% of coverage between the UPEC UTI89 and CFT073 genomes, as well as 4184 encoded proteins in common, and 724 and 1195 proteins exclusively found in UTI89 and CFT073, respectively. An specific genetic trait of UTI89 is the presence of a plasmid, pUTI89, of approx. 114 kb, which plays a role in virulence (Cusumano et al., 2010).

The Tn*5*-mutant library was constructed in the *E. coli* strain CFT073 and verified for random Tn-insertions across the bacterial genome (not shown). The library contained approx. 1.5×10^5 mutants. The previously generated library in *E. coli* UTI89, also tested in the current work, consisted of approx. 1.6×10^5 mutants (García et al., 2021).

3.2. Assessment of the input and output Tn-libraries and identification of essential genes in CFT073

Using TraDIS, the Tn*5* insertions sites and the flanking genomic regions across the genomes of UTI89 and CFT073 were sequenced and analyzed in the 20 libraries under study (Table 2). Details on the number of reads, % of reads that mapped to the UTI89 or CFT073 reference genomes, and number of Tn-UIS for each library are shown in Table 2. A total of 140,380 UIS were identified in the CFT073 input library when sequencing reads from duplicates of the library were combined, which corresponds to an average of one Tn-UIS every 37.3 bp across the genome of the strain (Table 2, Fig S1). Using the combined CFT073 input library, 457 genes were predicted as essential (Table S1).

According to the COG analysis (Fig. 2), six of the identified essential genes were categorized as belonging to two different COGs. Genes involved in translation, ribosomal structure and biogenesis (COG J) and cell wall/membrane/envelope biogenesis (COG M) represented the two most dominant categories (15.1% and 7.2%, respectively of the total number of essential genes identified). Other frequent categories encompassed genes involved in nucleotide transport and metabolism (4.16%) (COG F), lipid transport and metabolism (4.6%) (COG I), replication, recombination and repair (3.5%, respectively) (COG L) and transcription (3.1%) (COG K). A high proportion of the essential genes (49.4%) encoded proteins of unknown function, or the genes were not categorized as belonging to any COG.

3.3. Identification of CFT073 fitness genes during growth in human urine and comparison with previous studies

Previously, we used TraDIS to identify fitness genes in UTI89 during growth and survival in human urine (García et al., 2021). The results obtained differed from those obtained by Shea et al. where an ordered Tn-library was used to define fitness genes for UPEC CFT073 in human urine (Shea et al., 2020). For comparison purposes, in the current study, we tested the input Tn-library in CFT073 under similar urine growth conditions to those previously applied for the UTI89 Tn-library (García et al., 2021).

A total of 215 genes were predicted as fitness genes in CFT073 after 20 h of growth in human urine *versus* 64 genes previously found in UTI89 at the same time point (García et al., 2021) despite both strains showed similar growth patterns (not shown), and only 36 genes were shared between the pool of urine fitness genes in the two strains (Table S2, Fig S2). Compared to the previous study using an ordered Tn-library in UPEC CFT073 (Shea et al., 2020), of the 87 genes identified as fitness genes in human urine, only 12 were detected in the equivalent

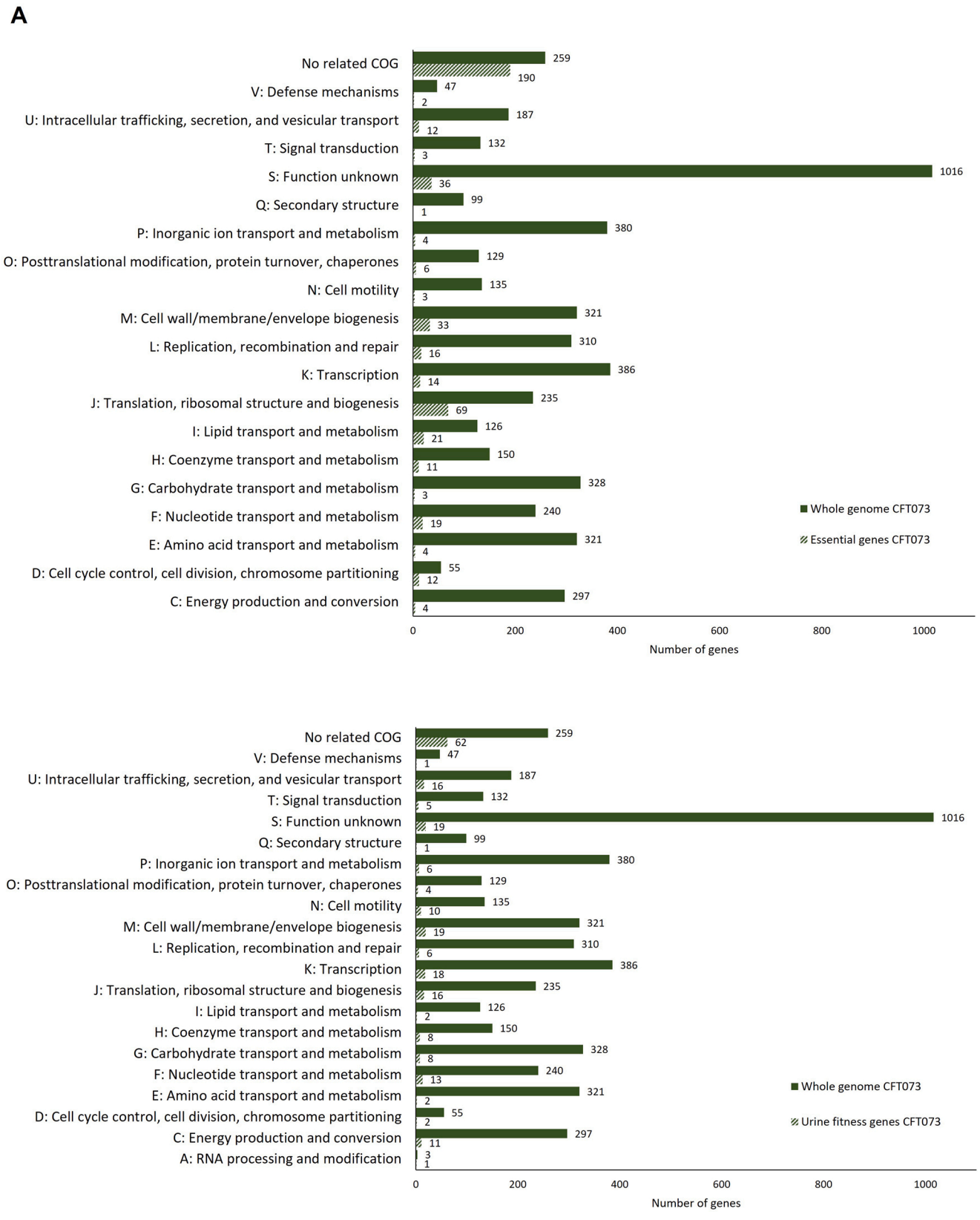

Fig. 2. Functional classification of essential and fitness genes in UPEC CFT073. Essential genes for growth in LB agar media supplemented with Kn (A) and fitness genes for growth in human urine (B) predicted in *E. coli* CFT073 were functionally categorized using the EggNOG database (illustrated on the vertical axis). The numbers indicate the essential/fitness genes in each functional category (striped bars) compared with the total number of genes in the reference strain and belonging to the same category (green bars).

list of fitness genes in our study (Table S2). In the current work, the gene *rfaC*, involved in LPS biosynthesis, showed the greatest difference between the output and input libraries (logFC of −10.3) in CFT073 (Table S2).

Results from the COG analysis revealed that in CFT073, dominant genes were associated with LPS biosynthesis (4.2%; including *rfa* genes, which were also identified in UTI89 (García et al., 2021), ATP synthesis (3.2%, all of which were previously detected in UTI89 (García et al., 2021), the Tol-Pal system (2.3% with 4 *tol* genes predicted in UTI89 (García et al., 2021), and genes encoding ribosomal functions (1.9%, with one gene shared with UTI89 (García et al., 2021) (Table S2). Besides this, the list of urine fitness genes in CFT073 encompassed 11 fimbriae-associated genes (including *fimB)* and three genes linked to iron uptake/transport (*hemD, hemE* and *tonB*). These genes have been previously reported as relevant for the growth of UPEC in human urine (Hagan et al., 2010; Shea et al., 2020), however, they were not among the urine fitness genes in UTI89 (García et al., 2021). The genes *purB* and *guaA,* involved in purine biosynthesis (Pang et al., 2012) were detected as fitness genes in both strains (Table S2) (García et al., 2021), which is in agreement with previous literature (Russo et al., 1996; Shea et al., 2020).

3.4. Identification of UTI89 and CFT073 fitness genes during UTI of the pig model

A pig UTI model has been recently developed and applied to test the infection ability of *E. coli* UTI89 (Nielsen et al., 2019; Stærk et al., 2021). Similar to mouse infections in our previous study (García et al., 2021), we inoculated the pigs with a dose of each input library of approx. 10^8-10^9 Tn-mutants (Table S3) and the output libraries were recovered after 6 h of infection from pig urine and bladders. CFUs detected for each sample are shown in Table S3. USG was within the average range encountered for pigs and humans (1.005 – 1.030) indicating a normal urine production rate at the time of inoculation (Table S3). Results from histology examinations showed the presence of diffuse cellular infiltration in the lamina propria and muscularis propria of the bladder tissue from three out of the four pigs tested at 6 hpi (Fig S3). The resulting output Tn-libraries contained UIS as far apart as every 36.8–93.2 bp (Table 2).

A total of 483 and 854 (826 chromosome- and 28 plasmid located) genes were scored as fitness genes during *in vivo* growth in pig urine in CFT073 and UTI89 respectively, while for bladder colonization, based on the composite libraries, 764 and 1257 (1213 chromosome- and 44 plasmid-encoded) genes were predicted as fitness genes in each of the strains (Table S4, Table S5). The genes *rfaC*, *rfaG* (both associated to LPS synthesis), *UTI89_C2023* (encoding a hypothetical protein) and *tolQ* (related to the Tol-Pal system) showed the greatest LogFC differences (10.38, −14.97, −12.73 and −13.11, respectively) between each of the output libraries (CFT073_pig_urineMIX, CFT073_pig_bladderMIX, UTI89_pig_urineMIX and UTI89_pig_bladderMIX, respectively) and the corresponding input (Table S4, Table S5).

The overall list of fitness genes for UTI in the pig model, defined as the set of common fitness genes between *in vivo* growth in pig urine and bladder colonization, included 439 and 741 (715 chromosome – and 26 plasmid-located) genes in CFT073 and UTI89, respectively (Table S4, Table S5). Of them, 188 and 42 were identified in the lists of fitness genes during *in vitro* growth in human urine in CFT073 and UTI89 (García et al., 2021), respectively (Table S4, Table S5, Fig S2). A total of 34 genes were common to the sets of fitness genes for pig UTI, growth in human urine and colonization of the mouse bladder (García et al., 2021) in UTI89 (Table 3, Fig S2).

At the single pig level, 1418 (UTI89_pig_bladder1), 1419 (UTI89_pig_bladder2) with 1049 genes in common, and 920 (CFT073_pig_bladder1) and 867 (CFT073_pig_bladder2) with 602 genes in common, were predicted as fitness genes during bladder colonization (Table S4, Table S5, Fig S2). In general, genes involved in LPS and ATP synthesis, members of the Tol-Pal system as well as fimbriae and flagella associated-genes, were found in all the lists of fitness genes, regardless of the host and the UTI-related condition (Table S4, Table S5).

The COG analysis of fitness genes during UTI of the pig model (Table S6) revealed that the majority of the genes were either not

Table 3
Common fitness genes between pig UTI, *in vitro* growth in human urine and mouse bladder colonization in *E. coli* UTI89 scored using TraDIS.

Gene_name	Product[1]	Main function (COG)
tolA[2,3]	Membrane spanning protein TolA	Protein transport/cell division/membrane integrity (U)
rfaF[4]	ADP-heptose-LPS heptosyltransferase 2	LPS biosynthesis (M)
degS[5]	Serine endoprotease DegS	Protease (O)
recC[4]	RecBCD enzyme subunit RecC	DNA repair (L)
rfaC	Lipopolysaccharide heptosyltransferase 1	LPS biosynthesis (M)
rfbC	dTDP-4-dehydrorhamnose 3,5-epimerase	LPS biosynthesis (M)
UTI89_C4945	Hypothetical protein	(NI)
wbdM	Putative glycosyltransferase WbdM	LPS biosynthesis (M)
rfaD	ADP-L-glycero-D-manno-heptose-6-epimerase	Carbohydrate metabolism (G, M)
UTI89_C1262 (ycfB)	tRNA-specific 2-thiouridylase MnmA	tRNA modification (J)
rpsI	30 S ribosomal protein S9	Translation (J)
atpF	ATP synthase subunit b	ATP synthesis (C)
yhbC	Ribosome maturation factor RimP	Ribosomal small subunit biogenesis (J)
iscS	Cysteine desulfurase	Transferase (E)
rpoE	RNA polymerase sigma factor	DNA binding (K)
topA	DNA topoisomerase I	DNA topological change(L)
csrA	Translational regulator CsrA	Translation regulation (J)
rfaG[2,5]	Lipopolysaccharide core biosynthesis glucosyltransferase	LPS biosynthesis (M)
UTI89_C4521	Hypothetical protein	(NI)
dnaT	Primosomal protein I	DNA replication (J)
UTI89_C3837	Hypothetical protein	(NI)
cydB	Cytochrome d terminal oxidase polypeptide subunit II	Oxidoreductase (C)
rplA	50 S ribosomal subunit protein L1	Regulation of translation (J)
wzxE[2]	Lipid III flippase	LPS biosynthesis (U)
dnaK	Chaperone protein DnaK	Protein folding (O)
atpB	ATP synthase subunit a	ATP synthesis (C)
yciM	Lipopolysaccharide assembly protein B	LPS biosynthesis (G)
ybeY	Endoribonuclease YbeY	rRNA processing/ribosome biogenesis (J)
galU[4]	UTP–glucose-1-phosphate uridylyltransferase	Galactose and UDP-glucose metabolism/ LPS biosynthesis (M)
icdA	Isocitrate dehydrogenase	Tricarboxylic acid cycle (C)
UTI89_C1265	Hypothetical protein	(NI)
purB[4]	Adenylosuccinate lyase	Purine metabolism (F)
corA[5]	Magnesium transport protein CorA	Cobalt, magnesium and nickel transport (P)
UTI89_C4804	HTH cro/C1-type domain-containing protein	DNA binding (K)

Genes are listed according to LogFC -from the lowest to the highest value- detected during mouse bladder colonization (García et al., 2021).
Genes also shown to be fitness genes in the common list (all conditions tested) for CFT073 are highlighted in bold.
[1]Gene products were re-annotated using the UniProt database.
[2]Genes previously identified as required for serum resistance of *E. coli* EC958 using TraDIS (Phan et al., 2013)
[3,4]Genes previously identified as required for growth/survival in human urine using different Tn-mutagenesis approaches (Hancock et al., 2008)[3], (Shea et al., 2020)[4].
[5]Genes previously demonstrated to play a role during colonization of the mouse bladder (Aguiniga et al., 2016; García et al., 2021).

assigned a COG (22.1% and 26.1% in CFT073 and UTI89, respectively) or encoded proteins of unknown function (15% and 21% in CFT073 and UTI89). Regarding known encoded functions, genes involved in cell wall/membrane/envelope biogenesis (8.2%), carbohydrate transport and metabolism (7.7%), transcription (7.5%), energy production and conversion (6.6%), intracellular trafficking, secretion, and vesicular transport (6.6%), cell motility (5.4%) and translation, ribosomal structure and biogenesis (5.2%) were the most commonly identified in CFT073. In UTI89, the most frequently detected categories included genes playing a role in transcription (8.1%), translation, ribosomal structure and biogenesis (5.4%), carbohydrate transport and metabolism (5.3%) and energy production and conversion (4.6%). Overall, the same COGs were identified among the fitness genes in both UPEC strains with the number of associated genes differing between them (Table S6).

Based on the composite libraries, 11 and 75 genes were classified as anti-fitness genes in CFT073 during *in vivo* pig bacteriuria and bladder colonization, respectively (Table S4). In UTI89, the equivalent list included 437 (pig urine) and 559 (pig bladder colonization) anti-fitness genes (Table S5).

3.5. Deletion of the gene rfaG yields UPEC growth attenuation during in vitro growth in pig and human urine as well as during UTI in the pig model

The gene *rfaG* has recently been demonstrated to play a role in UTI89 during growth in human urine and during colonization of the mouse bladder (García et al., 2021). In the present study, TraDIS analyses identified *rfaG* as a fitness gene during UTI in the pig model in both UPEC strains (Table S4, Table S5, Table 3) and during growth in human urine in CFT073 (Table S3). To validate these observations, the growth of defined mutants in *rfaG* was analyzed and compared to the growth of the corresponding WT strain under several UTI-related conditions. Results confirmed that the gene was important for growth in human urine in CFT073, as the WT significantly outcompeted the mutant in the urine assays (Fig S4). Furthermore, the gene was also proved to be relevant for *in vitro* growth in pig urine (Fig. 3) and for UTI of the pig model (Fig. 4, Table S7) in the two strains. Thus, the mutants CFT073Δ*rfaG* and UTI89Δ*rfaG* showed significant growth attenuation compared to the corresponding WT in pig urine *in vitro* (Fig. 3), and they were significantly outcompeted by the WT during growth in pig urine *in vivo* and cystitis at the time points tested (Fig. 4, Table S7). At 24 hpi, mutants lacking *rfaG* could not be recovered from urine or tissue-specimens in any of the animals (Fig. 4, Table S7).

Gross pathology of whole bladders removed at 24 hpi revealed distinct signs of inflammation in most bladders suggesting that the inoculation gave rise to localized UTI (Fig S5). WT CFT073 was also recovered from the right kidney of one pig, although in low numbers, while the mutant was not detected in any kidney. No kidney samples, however, showed macroscopic signs of inflammation (Fig S6). Differential cell count showed an insignificant change in neutrophil granulocytes in response to infection with neutrophil ratios (baseline *vs* 24 hpi) of 1.2 (Range: 0.85 – 1.39) and total blood neutrophile counts were within the normal range for pigs, suggesting only localized disease (*i.e.* in the bladder) without systemic inflammation.

As previously confirmed for UTI89Δ*rfaG* (García et al., 2021) by performing whole genome sequencing and bioinformatic analysis, the mutant CFT073Δ*rfaG* was confirmed to specifically lack the gene *rfaG*, which was replaced with a chloramphenicol selectable marker and no additional mutations were present in the strain. In addition, CFT073Δ*rfaG* and CFT073 Rif^R were proved to show similar growth to the WT in LB and EZ MOPS media (not shown).

4. Discussion

Fitness and virulence genes in UPEC required to cause UTI have been suggested to be strain- and host -dependent (García et al., 2021; Torres-Puig et al., 2022). Indeed, in our previous study, where we applied TraDIS to identify fitness genes in UTI89 during growth in human urine and during UTI in a mouse model, we revealed that, on one hand, fitness genes varied depending on the condition tested and that, on the other hand, variation was also observed from mouse to mouse. However, we also demonstrated that some factors, such as LPS biosynthesis, are important regardless of the host or the UTI-condition tested (García et al., 2021). Here, we applied TraDIS to predict fitness genes in CFT073 and UTI89 during infection of a recently developed porcine model of UTI (Nielsen et al., 2019), and we assessed strain-specific differences. To our knowledge, this is the first time that a large-animal model has been used for studies on genome wide-analysis during UTI. Notably, the pig UTI model may more accurately reflect disease conditions in humans than the mouse model since pigs and humans share anatomical and physiological properties, as well as a natural susceptibility to UTI (Stærk et al., 2022). Furthermore, the large size of pigs has also practical benefits allowing the collection of large quantities of biological material (*i.e.* urine, blood, tissue) and facilitating differentiation between sessile and planktonic UPEC subpopulations, which is not feasible in the murine models where urine specimens cannot be reliably collected in sufficient amounts.

For analysis of strain-specific factors, fitness genes in the strain CFT073 during growth in human urine were investigated using a similar TraDIS approach to this previously performed for UTI89 (García et al., 2021). Importantly, the gene *rfaG*, which is involved in LPS-biosynthesis, was shown to play a role during all the UTI-related conditions under study in both strains.

Essential genes for the growth of CFT073 were investigated as previously reported for UTI89 (García et al., 2021). Interestingly, the list of essential genes in CFT073 (N = 457) differed from those obtained in other *E. coli* strains. Thus, in our previous study, 293 genes were scored as essential for growth on LB agar plates with Kn in UTI89 (García et al.,

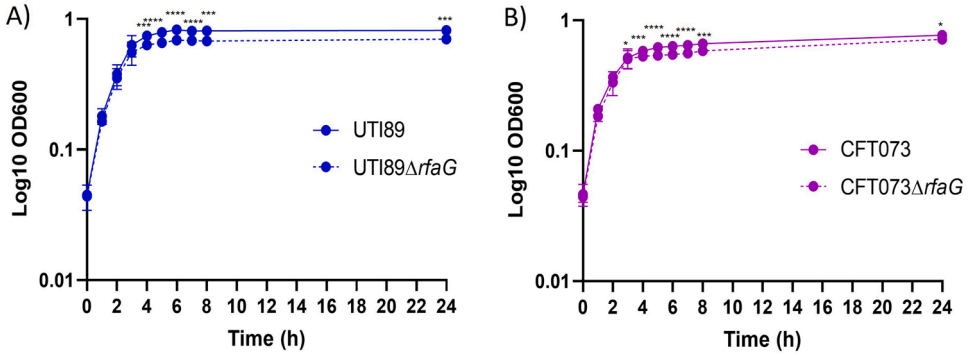

Fig. 3. Growth curves obtained for *E. coli* UTI89 and UTI89Δ*rfaG* (A), and *E. coli* CFT073 and CFT073Δ*rfaG* (B) in pig urine. The data shown are means ± standard deviations of at least three biological replicates. Statistical significance (****p < 0.0001; ***p < 0.001; *p < 0.05) was determined by one-sample t-test.

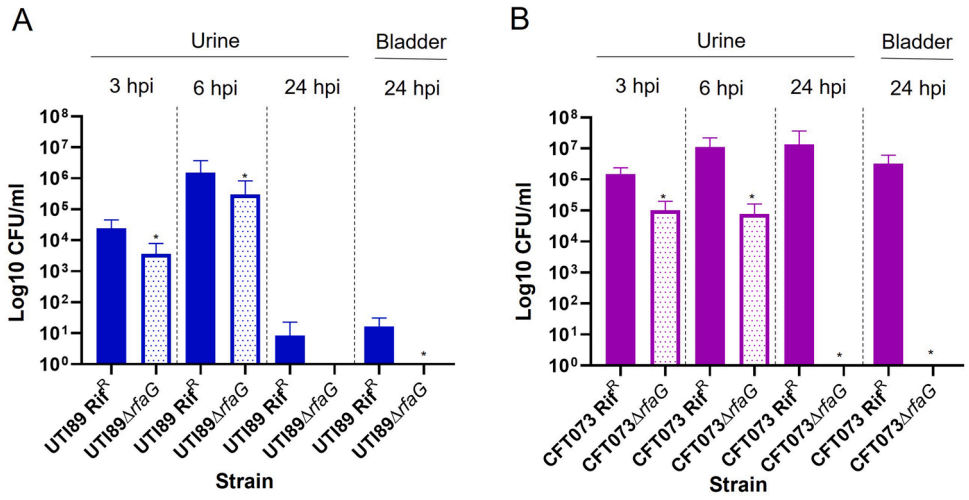

Fig. 4. Results derived from competition infection experiments in pigs. CFU counts obtained for UTI89 Rif^R and the derived mutant UTI89Δ*rfaG* (A) and CFT073 Rif^R and the derived mutant CFT073Δ*rfaG* (B) at 3, 6 and 24 hpi in urine and at 24 hpi in bladder. The data shown are means ± standard deviations of results obtained from three pigs. Statistical significance (*p < 0.05) was determined by one-sample t-test.

2021), and here, we showed, that 38.9% of them are exclusively found in this strain when compared to UPEC CFT073. Of the 457 genes identified in CFT073 in the current work, 202 (44.2%) and 187 (40.9%) were also essential in *E. coli* K12 and UPEC EC958 (Phan et al., 2013; Goodall et al., 2018). In general, the same COG groups were identified as essential in the three UPEC isolates, however, the number of protein-coding essential genes varied in each category between the strains, which suggests that similar functions may be essential for growth in every *E. coli* isolate. For example, COG M and COG J were the most frequently found categories in CFT073, UTI89 (García et al., 2021) and EC958 (Phan et al., 2013), and, other categories such as COG I, COG F, COG L, and COG K were also dominant in the three strains (Phan et al., 2013; Goodall et al., 2018; García et al., 2021).

Interestingly, the set of predicted fitness genes in CFT073 during growth in human urine (N = 215) differed from the one determined in UTI89 (N = 64) (García et al., 2021) using TraDIS, and only 10.8% of the CFT073 urine fitness genes were common to the list in UTI89. This indicates that differences in nutritional and other requirements for growth in urine are strain-dependent. Discrepancies observed may be attributed to differences in gene content. In accordance with this, comparative genomics revealed that 724 and 1195 proteins were unique to UTI89 and CFT073, respectively. Differences might also be due to differences in library saturation; UIS were identified at an average of 23.3 bp and 37.3 bp in each strain, with the UTI89 Tn-library being higher saturated than this generated in CFT073. Our CFT073 list of urine fitness genes also differed from the one obtained for the same strain and similar conditions in a previous report using an ordered Tn library in CFT073; only 13.8% of the genes found in that study (Shea et al., 2020) were identified in the current work. Neither amino acid nor peptide biosynthesis genes were found to be fitness genes for UPEC CFT073 during growth in human urine, which is in agreement with our previous work using UTI89 (García et al., 2021) but contradicts previous reports (Alteri et al., 2009; Sintsova et al., 2019; Shea et al., 2020). The differences detected might be attributed to the different approaches applied to identify CFT073 fitness genes during growth in human urine. Discrepancies *versus* previous studies might also be explained by the fact that urine seems to be a highly unstable medium. Different studies demonstrated that urine composition might not only differ from host to host but also from the time point where urine was collected. Thus, factors such as changes in diet and fluid intake might influence urine content, and, therefore, the results obtained, as previously suggested (García et al., 2021). Furthermore, urine density, which varies according to fluid intake and diet, has been shown to directly influence UPEC growth rate and morphological phenotype (Klein et al., 2015).

Overall, fimbriae-associated and iron-uptake genes, genes involved in purine, LPS and ATP synthesis as well as the Tol-Pal system were identified as relevant in CFT073 during growth in human urine, as previously reported (Hagan et al., 2010; Buckles et al., 2015; Aguiniga et al., 2016; Hirakawa et al., 2019; Shea et al., 2020; Torres-Puig et al., 2022).

The mouse model of UTI has been used for decades as the standard animal model to investigate UPEC determinants linked to UTIs (Hung et al., 2009). In our previous work, we employed a modified mouse model of UTI, which allowed us to assess gene fitness contribution in UPEC UTI89 when using TraDIS. In this model, the problem of the bottleneck effect when performing Tn-insertion sequencing studies was overcome, and the urine environment in that model was close to the one encountered in humans with a similar urine concentration (Walters et al., 2012; García et al., 2021). However, the intrinsic resistance against UTI in mice suggests that UTI in this animal may differ to some extent from UTI in humans, and thus, the identified fitness genes may be different from those in humans (Carey et al., 2016). Indeed, the genetic-expression response in mice models has been shown to poorly reflect human inflammatory disease (Seok et al., 2013). To assess fitness factors relevant to human UTI it is therefore essential that the animal model chosen reflects the disease in humans as much as possible. In this regard, pigs have been highlighted as an excellent animal model for studying human infectious diseases (Meurens et al., 2012). Especially human UTI pathogenesis is well-recapitulated in pigs, which are naturally susceptible to UTI caused by UPEC and other human-related pathogens (Belanger et al., 2011; Stærk et al., 2021). Therefore, it is reasonable to assume that UPEC fitness genes identified in the pig UTI model are likely also to be important during human UTI. In the present study, we investigated fitness genes in UTI89 and CFT073 during early (at 6 hpi - as for mice studies- (García et al., 2021)) *in vivo* bacteriuria and colonization of the pig bladder. Histology examinations of the bladders demonstrated that already after 6 h of infection, an inflammatory response could be observed in the organs, confirming that infecting the pigs with the input Tn-libraries resulted in manifested infection at this (short) timepoint. Analysis of the output pools from urine and bladder revealed a UIS every 36.8–93.2 bp which indicates a good saturation of the libraries. Specially, results from the output libraries from the bladder (UIS every 45.5–93.2 bp), confirms the robustness of the experimental design, and proves that the bottleneck effect due to urination and, therefore, stochastic loss of mutants in urine was at least partially overcome. Furthermore, our results showed that

UPEC multiplies very fast already after 6 h of infection in both niches since the number of mutants detected were surprisingly high in urine (approx. 10^8 CFU/mL) and in the bladder (approx. 10^4-10^5 CFU/mL) at this time point. Indeed, we can only make conclusions on fitness genes relevant during the early stage of infection (up to 6 h), and it is likely that other genes are important during prolonged infection. However, considering the bottleneck effect, prolonged infection protocols may not be feasible. The combined list of fitness genes in UTI89 in this model (N = 741), which represents fitness genes common to *in vivo* growth in pig urine and bladder colonization, differed from the published list obtained during mouse UTI (N = 590) (García et al., 2021), with 222 genes (29.9% and 37.6% of the pig UTI and mice UTI associated-genes, respectively) in common. Such results suggest an effect of the host environment on the contribution of individual genes to fitness. In addition, sets of fitness genes during UTI in the pig were strain-dependent (741 in UTI89 *versus* 439 in CFT073). In accordance with the previous observation from studies in mice (García et al., 2021), a rather large variation was observed between single animals. Similarly, transcriptomics studies on urine collected from women diagnosed with UTI, revealed that different genes were expressed depending on the donor (Hagan et al., 2010). These results point out that the UTI environment varies from host to host, and importantly it appears that UPEC evolution has involved, on one hand, assuring fitness in different niches within the same host, and on the other hand, allowing the microorganism to adapt to environmental differences between hosts (García et al., 2021). Taking into account these data, a recommendation is to carry out analysis of results using composite samples.

According to the results from the COG analysis, although the lists of fitness genes varied between the two strains, the associated functions relevant for UTI in the pig model might be the same regardless of the isolate. Furthermore, dominant functions during pig UTI, including; i) transcription, ii) translation, ribosomal structure and biogenesis, iii) carbohydrate transport and metabolism and, iv) cell wall/membrane/envelope biogenesis, v) energy production and conversion, appeared to be the same in both strains.

Contrary to results from the traditional mouse model of UTI (Alteri et al., 2009; Mao et al., 2012; Subashchandrabose et al., 2014), the genes *nik* (linked to nikel uptake), *edd* (gluconeogenesis), *speB* (putrescine biosynthesis), *nanA* (sialic acid metabolism), *cus* (copper tolerance system), *cjrABC-senB*, *shiA*, and *fbpB* (all the three involved in virulence), and osmotic stress related-genes, were not classified as fitness genes in the pig model in the current study. These genes were not either scored as fitness genes using TraDIS in UTI89 during UTI of the modified mouse model (García et al., 2021). In addition, the gene *cutA* (copper tolerance), and the genes *phnO* and *ypd* (associated to different metabolic pathways), which were previously shown to be required for infection of the mouse model of UTI in UTI89 (García et al., 2021), were not on the UTI89 list of fitness genes for UTI in the pig. Similarly, the gene *tusA* (sulfur acquisition) (Dahl et al., 2013), recently demonstrated to be important during growth of UTI89 in human urine (García et al., 2021) was not found in any set of fitness genes identified in this study. Interestingly, in disagreement with previous studies in mice, in which the importance of motility during UTIs was discarded (Russo et al., 1999; García et al., 2021), flagella related-genes (*flg, fli* and *flh* genes) were identified as contributing to the fitness of UTI89 and CFT073 during UTI in the pig model. Furthermore, flagella-associated genes were on the list of common fitness genes for *in vivo* growth in pig urine and bladder colonization in both UPEC strains. Thus, flagella synthesis and motility seem to be of relevance during UTI in the pig model and not in mice.

In agreement with results from the murine UTI model, fimbriae related-genes were detected in the lists of fitness genes obtained during UTI of the pig model in CFT073 and UTI89, as well as in the fitness-gene-set for growth of CFT073 in human urine. Surprisingly, pigs inoculated with a rather low dose of approx. 10^3 CFU UTI89 Rif^R or UTI89Δ*rfaG*, almost cleared the infection completely or yielded very low numbers of bacteria in urine and bladder, compared to previous reports where UTI89 was shown to consistently colonize pig bladders at either similar or lower inoculum doses (Stærk et al., 2021, 2022). In these previous pig studies, the inoculum was prepared by preincubating UTI89 in static LB cultures to optimize type-1 fimbrial expression. In contrast, in the current work the inoculum was prepared from colonies growing overnight on plates and, thus, type-1 fimbriae genes may not be fully expressed. Therefore, we used a higher inoculum dose for CFT073 and its mutant (10^6 CFU/mL), and this led to higher bacterial titers in urine and bladder as well as to distinct signs of tissue-inflammation suggesting rapid disease evolution in these animals. Taken together, these and previous (Stærk et al., 2021) results suggest that type-1 fimbrial expression is important for adhesion, although fimbriae role seems to be of higher relevance at lower inoculum doses.

As in the mouse model (García et al., 2021), potassium uptake was required for infection of the pig; particularly, *kdpE* was scored as a fitness gene during UTI of the pig model in UTI89, and capsule biosynthesis genes (*kps*) were found in the fitness-gene-sets in both strains during pig UTI. Previous reports showed that biofilm formation is relevant for mouse and pig bladder colonization (Timmermans and Van Melderen, 2010; Nielsen et al., 2019). Accordingly, the curli related-gene, *csrA,* previously identified as a fitness factor during colonization of the murine bladder (García et al., 2021) was also a fitness gene in the pig model in UTI89. However, the gene was not scored as a fitness gene in CFT073, most likely due to the stringent criteria applied. Thus, it showed a logFC of − 5.39 and a Q-value of 0.003 in the fitness gene-set obtained from bladder colonization. Further studies are needed to understand whether biofilm formation plays an important role during UTI in the pig model, and probably longer infection times will be needed to study this.

Other genes scored as relevant for the outcome of UTI in our study in the two strains, such as *degS*, and *surA,* encoding a serine protease and an isomerase, respectively, have been also demonstrated to play a role during infection of mice (Redford et al., 2003; Justice et al., 2006). As previously shown for mice (Subashchandrabose et al., 2014), ethanolamine metabolism, was also predicted to be relevant for host colonization during pig UTI but only in UTI89. Other genes shown to play a role during UTI in mice in our previous study on TraDIS and UTI89 (García et al., 2021) such as *atpF* (ATP synthesis), *himD* (DNA recombination-bladder colonization), *wzxE* (polysaccharide transport), *ftsE* (cell division) and *tam* (metabolism) were all classified as fitness genes during pig UTI in this study in the two strains. Interestingly, the *corA* gene involved in the transport of magnesium, predicted in all fitness-gene-lists in this work, was previously shown to play a role during UTI of the mouse model in UTI89 (García et al., 2021). Therefore, magnesium may be scarce during UTI also in pigs. Six and 63 genes encoding hypothetical proteins were found in the lists of fitness genes for UTI89 (shared between the two animal models and human urine) and CFT073 (shared between the pig model and human urine). Thus, it would be of interest to further investigate the role of such factors during UTI.

It is important to emphasize that most of the fitness genes for growth in human urine were present in the lists obtained in pigs, with 87.8% and 95.3% of the genes in CFT073 and UTI89, respectively. The high number of common fitness factors between human and pig UTI could reflect the similarities of the two species, such as urinary tract anatomy, immune physiology and urine composition (Stærk et al., 2021). The latter is also supported in a study showing pig urine densities within the range of humans – in contrast to mice that produce highly concentrated urine outside the range of humans and pigs (Parfentjev and Perlzweig, 1933).

Common genes between all the lists of fitness genes in UTI89 and CFT073 included factors associated to LPS, purine and ATP synthesis, the Tol-Pal system, carbohydrate metabolism, the TCA cycle and transport of magnesium. Overall, aerobic respiration was predicted as relevant during UTI as reported (Alteri and Mobley, 2012, 2015; García et al., 2021). These factors have previously been demonstrated to be important for UPEC to cause UTI (Phan et al., 2013; Hirakawa et al.,

2019). Therefore, all results taken together indicate that such factors may be relevant during UTIs regardless of the strain, the condition or the host tested. This points out that some growth limitations for UPEC are common to the murine UTI model, the pig UTI model and UTI in humans, and such factors may represent useful targets for the development of new therapeutics against UTIs caused by UPEC. Among the common fitness factors, the gene *rfaG,* encoding for a glucosyltransferase involved in the synthesis of the LPS (surface component linked to adhesion) core region (Parker et al., 1992), seems especially promising. This gene has been identified in all the fitness-gene-lists obtained when applying TraDIS under different UTI-related conditions and regardless of the strain tested or the host (Phan et al., 2013; Shea et al., 2020; García et al., 2021). Moreover, the gene has been demonstrated to play a role during; i) growth of CFT073 (this work) and UTI89 (García et al., 2021) in human urine, ii) growth of UPEC EC958 in human serum (Phan et al., 2013) and, iii) UTI of the mouse (UTI89 and UPEC NU14) (Aguiniga et al., 2016; García et al., 2021), and the pig (UTI89 and CFT073) model (this work). In UPEC NU14, deletion of *rfaG*, also attenuated bacterial adherence to urothelial cells *in vitro* (Aguiniga et al., 2016). Besides, the gene was associated with virulence of *E. coli* and *Salmonella* in several reports (Genevaux et al., 1999; Lu et al., 2012; Deditius et al., 2015). Thus, in a previous study, *E. coli* K12 Tn10 insertion mutants in *rfaG* showed a decrease in adhesion to abiotic surface, displayed a deep-rough phenotype and showed a decreased 1 fimbriae production and motility compared to WT (Genevaux et al., 1999). Also, the gene has been confirmed to play a role in motility in *S.* Typhimurium (Deditius et al., 2015) and its absence in *S.* Pullorum yielded attenuation of virulence during infection of chickens (Lu et al., 2012). All together: decreased fimbria production and motility, decreased adhesion to urothelial cells, lack of RfaG glycotransferase activity -involved in oligosaccharide biosynthesis-, in the mutant compared to WT, may explain the observed phenotypes during UTI; *i.e.* growth defect in urine and attenuated infection of the bladder.

Overall, all these data support *rfaG* as a promising, potential target for new prophylactic or treatment strategies to combat UTIs.

In conclusion, this work has identified UPEC fitness genes during UTI of a pig model, which has not been previously described in the literature. Using TraDIS and the porcine UTI model, we identified a list of common fitness genes -including the LPS biosynthesis gene, *rfaG*- between several steps of a UTI and across the two strains tested. However, the set of fitness genes differed depending on the UPEC isolate, just as the set of human urine fitness genes and essential genes for growth varied considerably between the two strains.

CRediT authorship contribution statement

Vanesa García: Conceptualization, Investigation, Methodology, Validation, Visualization, Formal analysis. **Kristian Stærk**: Methodology, Investigation, Formal analysis, Visualization. **Mosaed Saleh A. Alobaidallah**: Methodology, Visualization, Data curation. **Rasmus B. Grønnemose:** Methodology, Formal analysis. **Priscila R. Guerra:** Methodology, Visualization. **Thomas E. Andersen**: Conceptualization, Resources, Formal analysis. **John E. Olsen:** Formal analysis, Resources, Writing – review & editing. **Ana Herrero-Fresno:** Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing, Funding acquisition, Supervision.

Conflict of interest

The authors have declared no conflict of interest.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127202.

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