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Decreased Expression of Type 1 Fimbriae by a *pst* Mutant of Uropathogenic *Escherichia coli* Reduces Urinary Tract Infection

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The *pstSCAB-phoU* operon encodes the phosphate-specific transport system (Pst). Loss of Pst constitutively activates the Pho regulon and decreases bacterial virulence. However, specific mechanisms underlying decreased bacterial virulence through inactivation of Pst are poorly understood. In uropathogenic *Escherichia coli* (UPEC) strain CFT073, inactivation of *pst* decreased urinary tract colonization in CBA/J mice. The *pst* mutant was deficient in production of type 1 fimbriae and showed decreased expression of the *fimA* structural gene which correlated with differential expression of the *fimB*, *fimE*, *ipuA*, and *ipbA* genes, encoding recombinases, mediating inversion of the *fim* promoter. The role of *fim* downregulation in attenuation of the *pst* mutant was confirmed using a *fim* phase-locked-on derivative, which demonstrated a significant gain in virulence. In addition, the *pst* mutant was less able to invade human bladder epithelial cells. Since type 1 fimbriae contribute to UPEC virulence by promoting colonization and invasion of bladder cells, the reduced bladder colonization by the *pst* mutant is predominantly attributed to downregulation of these fimbriae. Elucidation of mechanisms mediating the control of type 1 fimbriae through activation of the Pho regulon in UPEC may open new avenues for therapeutics or prophylactics against urinary tract infections.

Pathogenic *Escherichia coli* comprises a diversity of strains associated with both intestinal and extraintestinal infections (39). Urinary tract infections (UTIs) are one of the most common bacterial infections, and uropathogenic *E. coli* (UPEC) is the predominant causal agent, representing up to 85% of community-acquired UTIs (28). In addition to causing UTIs, extraintestinal pathogenic *E. coli* (ExPEC) is an important pathogen associated with neonatal meningitis and septicemia in humans, as well as systemic infections in poultry and livestock (60, 61). Many virulence factors associated with UPEC strains are important for establishing infection, and these include adhesins, toxins, iron acquisition systems, and capsular antigens (53).

An important aspect of bacterial virulence is the capacity to rapidly adapt to changes and stresses encountered during infection of the host, since changes in the host environment may serve as cues mediating regulation of expression of key virulence factors during infection (25, 47). One of the mechanisms by which bacteria respond to environmental signals is through two-component signal transduction systems (TCSs). TCSs are composed of an inner-membrane histidine kinase sensor protein and cytoplasmic response regulator (81). TCSs are important for bacterial adaptation and virulence (6, 12), and a number of TCSs have been identified to be important for pathogenic *E. coli*, e.g., BarA-UvrY, PhoPQ, and QseBC (4, 31, 41, 56).

The Pho regulon is controlled by the PhoBR TCS, in which PhoR is the sensor histidine kinase and PhoB the response regulator. PhoBR responds to phosphate limitation, i.e., when the extracellular phosphate concentration falls below 4 μM (36, 79). Under phosphate-limiting conditions, PhoBR induces genes belonging to the Pho regulon, which are involved in phosphate transport and metabolism (36, 79). The *pstSCAB-phoU* operon belongs to the Pho regulon and encodes the phosphate-specific transport (Pst) system, an ATP-binding cassette (ABC) transporter specific for inorganic phosphate (P_i). Mutations in the *pst* operon mimic phosphate starvation conditions and result in constitutive expression of the Pho regulon regardless of environmental phosphate availability (79). In addition to mimicking phosphate starvation, inactivation of the Pst system attenuated virulence of both pathogenic ExPEC and enteropathogenic *E. coli* (EPEC) strains (7, 15, 19, 24, 42). In UPEC, inactivation of *phoU*, a gene of the *pst* operon involved in repression of the Pho regulon under phosphate-replete conditions (36), reduced colonization of the murine urinary tract (5, 11). However, the precise role of the Pst system in UPEC virulence has not yet been investigated. Nevertheless, in avian pathogenic *E. coli* (APEC), attenuation of a *pst* mutant was attributed to increased sensitivity to serum, acid, oxidative stress, and cationic antimicrobial peptides (7, 20, 42, 44). Also, an altered membrane homeostasis and a reduction in the production of the hexa-acyl pyrophosphate form of lipid A are observed in this mutant strain (43, 44). Taken together, these results suggest that inactivation of the *pst* system could attenuate pathogenic *E. coli* by altering membrane composition and repressing production of surface structures, including adhesins required for colonization of host tissues. Indeed, inactivation of the *pst* system also inhibited expression of type 1 fimbriae by avian pathogenic *E. coli* (7, 20). However, the specific mechanisms by which induction of the Pho regulon inhibit expression of type 1 fimbriae have not been previously investigated.

Type 1 fimbriae mediate attachment of *E. coli* to host cells and

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tissues by mannose-specific receptors. Thereby, type 1 fimbriae are required to establish infection of ExPEC strains (34, 53). In UPEC strains, type 1 fimbriae are expressed in the bladder, mediate bacterial adherence to urothelial cells, and promote formation of intracellular bacterial communities (46, 66, 68, 85). The latter allows the persistence of UPEC strains in the host (34). Type 1 fimbriae are encoded by the *fimAICDFGH* operon (*fim*), where *fimA* encodes the major subunit and *fimH* encodes the mannose-specific adhesin (55). Expression of the *fim* operon is governed by a phase-variable promoter (*fimS*) which is located on a 314-bp invertible element flanked by two 9-bp inverted repeats (3). Phase-variable expression of type 1 fimbriae is mediated by the inversion of *fimS* to and from the "on" or "off" orientation. Orientation of *fimS* is controlled by the FimB and FimE recombinases (40). In this manner, FimE promotes switching of *fimS* to the off orientation (from phase on to phase off), while FimB mediates switching in both directions (from phase off to phase on and phase on to phase off), where the on orientation is favored (26, 40). In addition to FimB and FimE, the CFT073 UPEC strain encodes FimBE-like recombinases, IpuA and IpbA (10). IpuA promotes switching in both directions, like FimB, whereas IpbA promotes switching only in the on position (10). Furthermore, IpuA and IpbA are sufficient for switching *fimS* and influencing type 1 fimbriae expression either *in vitro* and *in vivo* (10, 33).

Inactivation of the Pst system has been shown to cause pleiotropic effects and loss of membrane integrity in APEC strains (7, 20, 42–44). However, the precise role of the Pst system in UPEC virulence has not been assessed. In this study, we investigated to what extent the Pst system affects UPEC virulence. We demonstrate that decreased expression of the type 1 fimbriae is directly linked to attenuated virulence of the *pst* mutant in the murine UTI model. Indeed, in the *pst* mutant, type 1 fimbriae are downregulated *in vitro* and *in vivo*. This downregulation is concomitant with differential expression of the recombinase genes *fimB* and *fimE* and the *fim*-like recombinases *ipuA* and *ipbA*. Furthermore, the *pst* mutant has a decreased capacity to invade bladder cells. The altered phenotypes of the *pst* mutant are due to the decreased expression of type 1 fimbriae, since constitutive expression of the *fim* operon restored these phenotypes. Therefore, our work shows that deletion of the Pst system in UPEC CFT073 reduces colonization of the bladder and invasion of uroepithelial cells specifically by reducing the expression of type 1 fimbriae.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth at 37°C and in human urine. Urine was collected from healthy female volunteers of ages 20 to 40 who had no history of UTI or antibiotic use in the prior 2 months. Each urine sample was immediately filter sterilized (0.2-μm pore size), pooled, and frozen at −80°C and was used within 2 weeks. 5637 bladder cells were grown in RPMI 1640 medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/liter glucose, and 1.5 g/liter sodium bicarbonate. Bacteria were also grown in morpholinepropanesulfonic acid (MOPS) minimal medium (Teknova) supplemented with 0.4% glucose, 0.2% $(NH_4)_2SO_4$, 1.32 mM K_2HPO_4, and 1 μg/ml thiamine (high phosphate [HP]). Low-phosphate (LP) MOPS contained 1 μM K_2HPO_4 (9). Antibiotics and reagents were added as required at the following concentrations: kanamycin, 40 μg/ml; ampicillin, 100 to 200 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 15 μg/ml; amikacin sulfate salt, 250 μg/ml; diaminopimelic acid (DAP), 50 μg/ml; and 5-bromo-4-chloro-3-indolylphosphate disodium (XP), 40 μg/ml.

Construction of mutants and complemented strains. All mutants were generated by the procedure described by Datsenko and Wanner using plasmids pKD3 and pKD13 as the template for chloramphenicol and kanamycin resistance cassettes, respectively (22). Primers used are listed in Table S1 in the supplemental material. Antibiotic cassettes flanked by FLP recombination target (FRT) sequences were removed by transforming the mutant strains with pCP20 expressing the FLP recombinase (16).

The Δ*pstSCA* strain was complemented by inserting the *pstSCA* genes at the *att*Tn*7* site of the chromosome. The procedure was performed as described by S. Crépin, J. Harel, and C. M. Dozois (submitted for publication). Briefly, the *pstSCA* genes and their native promoter were amplified with the primers CMD1069 and CMD1070. The amplified product was then digested with XhoI and XmaI and ligated into the multiple-cloning site (MCS) of the mini-Tn*7*-containing vector pGP-Tn7-Gm, generating the vector pGP-Tn7-*pst*. Strain MGN-617 (pGP-Tn7-*pst*) was conjugated overnight with the Δ*pst* strain, containing the vector pSTNSK, encoding the transposases *tnsABCD*, required for transposition of Tn*7* at the *att*Tn7 site, at 30°C on LB agar plates supplemented with DAP. After incubation, the bacterial lawn was suspended in 1 ml of phosphate-buffered saline (PBS), washed twice in PBS, serially diluted, spread on LB agar supplemented with gentamicin, and incubated at 37°C. Colonies were verified for sensitivity to kanamycin and ampicillin, indicating the likelihood of integration at *att*Tn7 and loss of the transposase-encoding plasmid pSTNSK. Insertion of Tn*7* into the *att*Tn7 site was verified by PCR using the primers CMD1070 and CMD1072 (see Table S1). The *pst*-complemented derivative was designated QT2117.

For the *fim* L-ON derivative strains, primers CMD1185 and 1186 (see Table S1 in the supplemental material) were used to amplify the *cat* cassette from pKD3. Using the same approach described by Gunther et al. (30), point mutations in primer CMD1186 were introduced in order to block the promoter switch in the on position.

Experimental UTI in CBA/J mice. Experimental infections were carried out using either competitive coinfection or single-strain infection models as described previously (32, 62). Prior to inoculation, strains were grown for 16 h at 37°C with shaking (250 rpm) in 55 ml of LB medium. For coinfection, cultures were centrifuged and pellets of the wild-type (WT) and derivative strains were mixed 1:1. Six-week-old CBA/J female mice were transurethrally inoculated with 20 μl of the 1:1 mixture containing 5×10^8 CFU of the UPEC CFT073 Δ*lacZYA* strain (QT1081) and 5×10^8 CFU of either the CFT073 Δ*pstSCA* (QT1911) strain or its complemented derivative (QT2117). At 48 h postinfection (p.i.), mice were euthanized; bladders and kidneys were aseptically removed, homogenized, diluted, and plated on MacConkey agar to determine bacterial counts.

In the single-strain experimental UTI model, mice were infected as described above but with a pure culture (10^9 CFU) of each strain, and at 6, 24, and 48 h p.i., bacterial counts were determined from the bladders and kidneys. Bladders were bisected; one half was used to determine bacterial counts and the other half was resuspended in TRIzol reagent (Invitrogen) for RNA extraction and subsequent analysis of bacterial gene expression.

Yeast cell aggregation and MRHA assay. A yeast cell aggregation assay was performed as described elsewhere (20). Briefly, cultures were grown at 37°C in LB broth or human urine to mid-log phase or for 24 h to 48 h in LB broth without shaking, to enhance expression of type 1 fimbriae. An initial suspension of approximately 2×10^{11} cells ml^{-1} in phosphate-buffered saline (PBS) was serially diluted 2-fold in microtiter wells, and equal volumes of a 3% commercial yeast suspension in PBS were added to each of the wells. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive aggregation reaction. The mannose-resistant hemagglutination (MRHA) assay was determined using O^- human red blood cells with bacteria grown to mid-log phase in LB broth. To

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
BW25113	F$^-$ lambda$^-$ (*lacI*^{q} *rrnBT14 lacZWJ16 hsdR514 araBADAH33 rhaBADLD78*)	22
CF1693	MG1655 Δ*relA251*::*km* Δ*spoT207*::*cm*; Km^r Cm^r	87
CF5747	MG1655 pALS13; Ptac -*relA′* (RelA 1-455), active RelA; Ap^r	76
CFT073	UPEC wild-type pyelonephritis strain	49, 80
χ7122	Avian pathogenic; O78:K80:H9; *gyrA* Nal^r	58
K3	χ7122 Δ*pstCAB*::*kan*; *Km^r*	42
MG1655	F$^-$ lambda$^-$ *ilvG rfb-50 rph-1*	8
MGN-617	*thi thr leu tonA lacY glnV supE* Δ*asdA4 recA*::RP4 2-Tc::Mu [pir]; Km^r	23
ORN172	*thr-1 leuB thi-1* Δ(*argF-lac*)*U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44* Δ(*fimBEACDFGH*)::*kan pilG1*; Km^r	84
QT1081	CFT073 Δ*lacZYA*::FRT	62
QT1324	CFT073 Δ*oxyR*::Km; Km^r	This study
QT1891	CFT073 Δ*pstSCA*::km; Km^r	This study
QT1911	CFT073 Δ*pstSCA*::FRT	This study
QT2063	CFT073 Δ*phoB*::FRT	This study
QT2085	MGN-617 + pGP-Tn*7*-*pst*	This study
QT2117	QT1911::Tn7T-Gm::*pstSCA*	This study
QT2138	CFT073 Δ*fimAICDFGH*::km; Km^r	This study
QT2207	QT1911 + pSTNSK	This study
QT2285	CFT073 *fimS* phase L-ON	This study
QT2305	QT1911 *fimS* phase L-ON	This study
QT2356	CFT073 + pKEN2	This study
QT2357	QT1911 + pKEN2	This study
S17-1(λpir)	λ*pir* lysogen of S17.1 (Tp^r Sm^r *thi pro hsdR$^-$ M$^+$ recA* RP4::2-Tc::Mu-*km*::Tn*7*)	65
Salmonella enterica serovar Typhimurium SL1344	*rpsL hisG*	29
Plasmids		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	22
pGP704	oriR6K mobRP4; Ap^r	48
pGP-Tn7-Gm	pGP704::Tn7T-Gm; Ap^r Gm^r	This study
pSTNSK	pST76-K::*tnsABCD*; Km^r	This study
pGP-Tn*7*-*pst*	pGP-Tn*7*-Gm::*pstSCA*	This study
pKD3	Template plasmid for the amplification of the *cat* gene bordered by FRT sites	22
pKD13	Template plasmid for the amplification of the *km* cassette bordered by FRT sites	22
pKD46	λ-Red recombinase plasmid Ts replicon; Ap^r	22
pKEN2	High-copy-no. phagemid constitutively expressing GFP; Ap^r	18
pST76-K	oriSC101 ts; Ap^r Km^r	57
pTNS2	oriR6K; encodes TnsABC+D specific transposition pathway; Ap^r	17
pUC18-mini-Tn7-Gm	Gm^r on mini-Tn*7*; for gene insertion in Gm^s bacteria	17

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm, streptomycin resistance; Tp^r, trimethoprim resistance.

inhibit type 1 fimbria-dependent hemagglutination, a final concentration of 1.5% α-D-mannopyranose was added to samples.

RNA extraction and quantification of gene expression. RNAs were extracted from bacterial cultures grown in LB broth or human urine, using the RiboPure-Bacteria kit (Ambion, Austin, TX), according to the manufacturer's recommendations, with the exception that DNase I treatment was performed twice. RNA was also extracted from infected bladders at 6, 24, and 48 h p.i. with TRIzol reagent (Invitrogen), followed by DNase 1 treatment (Ambion).

The iScript One-Step reverse transcription-PCR (RT-PCR) kit with SYBR green (Bio-Rad) was used for quantitative RT-PCR (qRT-PCR) according to the manufacturer's instructions. The *tus* gene was used as a housekeeping control (20). Each qRT-PCR run was done in triplicate, and for each reaction the calculated threshold cycle (C_T) was normalized to the C_T of the *tus* gene amplified from the corresponding sample. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (45). Genes with a fold change above or below the defined threshold of 2 are considered differentially expressed. Primers used for qRT-PCR analysis are listed in Table S1 in the supplemental material.

Detection and quantification of the on/off state of the *fimS* region. Detection of the orientation of the *fimS* region was performed as described previously (50, 73). Briefly, the *fimS* region was PCR amplified with the primers CMD1258 and CMD1259 (see Table S1), giving a fragment of 650 bp. The PCR products were digested with HinfI and analyzed on a 2% agarose gel. Profiles of the *fimS* switch are dependent on its orientation, with the on orientation producing fragments of 128 bp and 522 bp and the off orientation generating fragments of 411 bp and 239 bp. Quantification of the percentage of "on" cells was performed as described by Wu and Outten (86). The WT strain was cultured statically for 48 h at 37°C to enhance production of type 1 fimbriae and was used as a control of the on position. The WT strain was also cultured for 24 h on LB agar plates at room temperature and was used as a control of the off position.

Quantification of the orientation of the *fimS* switch was also performed by quantitative PCR (qPCR) with iQ SYBR green supermix (Bio-

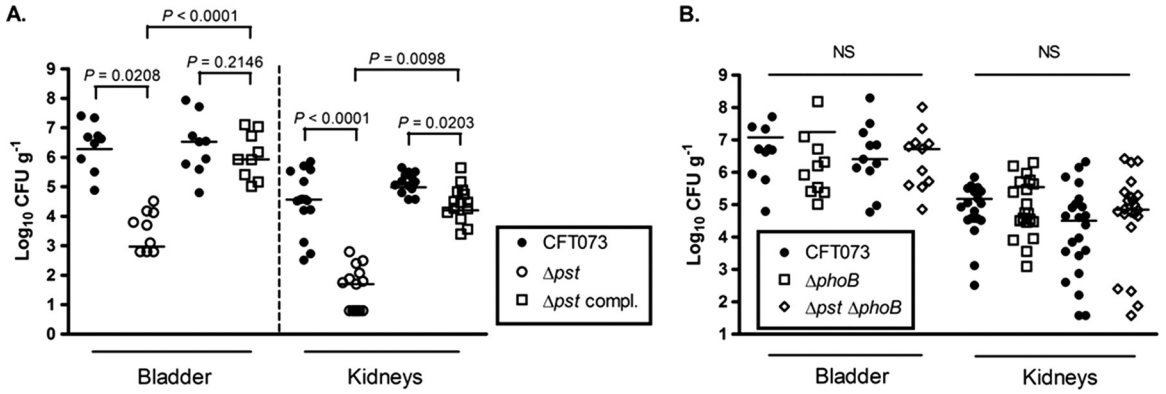

FIG 1 Inactivation of *pst* in uropathogenic *E. coli* CFT073 reduces competitive colonization of the mouse urinary tract. (A) CBA/J mice were coinfected with a 1:1 ratio of CFT073 Δ*lac* and either the Δ*pst* mutant or the Δ*pst* complemented (Δ*pst* compl.) strain. (B) CBA/J mice were coinfected with CFT073 Δ*lac* and CFT073Δ*phoB* or CFT073 Δ*pst* Δ*phoB*. Results are presented as the $\log_{10}$ CFU g^{-1}. Each data point represents a sample from an individual mouse, and horizontal bars indicate the medians. Each kidney was sampled separately. A Wilcoxon signed-rank test (two-tailed) was used to determine statistical significance. The Mann-Whitney test was used to determine the statistical differences between the *pst* mutant and the complemented derivative strain.

Rad) according to the manufacturer's recommendations. qPCR was carried out with 10 ng of genomic DNA (gDNA) extracted from bacteria grown under different culture conditions as described above or from 500 ng of total DNA extracted from infected bladder as described by Struve and Krogfelt (75). Primers CMD1246 and CMD1248 were used to amplify the on orientation, while the CMD1247 and CMD1248 primers amplified the off orientation. The C_T of the on and off orientations was normalized to the C_T of the *vat* gene (CMD96 and CMD97), an uninvertible element. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method (45).

Preparation of fimbrial extracts and Western blotting. Preparation of fimbrial extracts and Western blotting were performed as described previously (20), with anti-FimA serum from *E. coli* strain B_{AM} and F1C fimbria-specific (anti-$F165_2$) antiserum (35).

Nucleotide extraction and quantification of ppGpp. Nucleotides were extracted essentially as described by Traxler et al. (77), with the exception that bacteria were grown for 16 h or to mid-log phase of growth in 20 ml of LB medium. ppGpp was quantified by an Äkta purifier system using a Mono Q 5/50 GL column (GE Healthcare). The chromatography conditions were according to the method of Traxler et al. (77), with the exception that ppGpp was identified as a peak that eluted at ~54.6% of 1.5 M sodium formate buffer. Samples were run in duplicate for at least three separate time course experiments. The ppGpp standard was purchased from TriLink Biosciences. Detection of ppGpp was in the range of 20 nM to 100 mM. The CF5747 strain, which contains a *relA* inducible vector, and CF1693, where the *relA* and *spoT* genes were deleted, were used as positive and negative controls, respectively (76, 87).

Adhesion and invasion assays. The human bladder epithelial cell line 5637 (American Type Culture Collection HTB-9) was grown to confluence in 24-well plates in RPMI 1640. UPEC CFT073 and its derivative strains were grown in LB medium at 37°C to the mid-log phase of growth (optical density [OD] of 0.6). The bacterial cells were centrifuged, washed twice with PBS, resuspended in RPMI 1640 medium (Wisent Biocenter, St-Bruno, Canada) supplemented with 10% fetal bovine serum at 10^6 CFU ml^{-1}, and added to each well. Bacterium-host cell contact was enhanced by a 5-min centrifugation at 600 × *g*. For invasion assays, at 2 h postadhesion, cells were washed three times and a 2-h treatment with amikacin sulfate (250 μg/ml) was performed to kill extracellular bacteria. At 2 h posttreatment, cells were washed three times and lysed with PBS–0.1% sodium deoxycholate (DOC), serially diluted, and plated on LB agar plates. Quantification of cell-associated bacteria and intracellular bacteria (via an amikacin protection assay) was performed as previously described (46). To block adherence mediated by type 1 fimbriae, 1.5% α-D-mannopyranose was added to culture medium.

Resistance to serum, oxidative stress, polymixin B, and vancomycin. Resistance to 90% human serum was determined as described by Lamarche et al. (42). The serum was pooled from three independent persons not working with *E. coli*. A strain was considered resistant if there was an increase in bacterial counts or a decrease of less than 2 logs and sensitive if there was a decrease of more than 2 logs. Resistance to oxidative stress was performed as described by Crépin et al. (20). Differences in resistance were assessed from the diameter of the inhibition zone of strains cultured to the mid-log phase of growth in LB medium. Resistance to polymixin B and vancomycin was determined as described by Lamarche et al. (44), with slight modifications. Strains were grown to the mid-log phase of growth in LB broth, and then 10^6 CFU ml^{-1} was mixed, in microwell plates, with final concentrations of polymixin B (0.4 to 0.6 μg ml^{-1}) or vancomycin (50 to 100 μg ml^{-1}), and were incubated for 3 h at 37°C. Bacterial growth was evaluated by spectrophotometry. The MIC was considered the lowest drug concentration that reduced growth by >50% compared with growth in the absence of antibiotic.

Electron microscopy. Electron microscopy was performed as described previously (20) with bacterial cultures grown in LB broth to mid-log phase.

Statistical analyses. All data were analyzed by using the GraphPad Prism 4 software program. A Wilcoxon signed-rank test (two-tailed; $P \leq 0.05$) was used to determine statistical significance for coinfections. In single-strain infection experiments, a Mann-Whitney test was used. All other statistical analyses were determined by the Student *t* test or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test.

RESULTS

The *pst* mutant was outcompeted in a murine UTI cochallenge model. To characterize the role of the Pst system in UPEC strain CFT073, coinfection in the mouse UTI model was performed between the Δ*pstSCA* mutant (*pst* mutant) and the WT CFT073 Δ*lac* strain or between CFT073 Δ*lac* and the complemented strain (Δ*pst* compl.). The CFT073 Δ*lac* strain is as virulent as the CFT073 wild-type parent and presented no statistical difference in urinary tract colonization (62). At 48 h postinfection (p.i.), the *pst* mutant was outcompeted 732-fold in bladder and 2,649-fold in kidneys (Fig. 1A). Complementation with the *pstSCA* genes into the *att*Tn7 site significantly improved the competitive fitness in both the bladder and kidneys (Fig. 1A). The complemented strain regained competitive colonization in the bladder and kidneys, al-

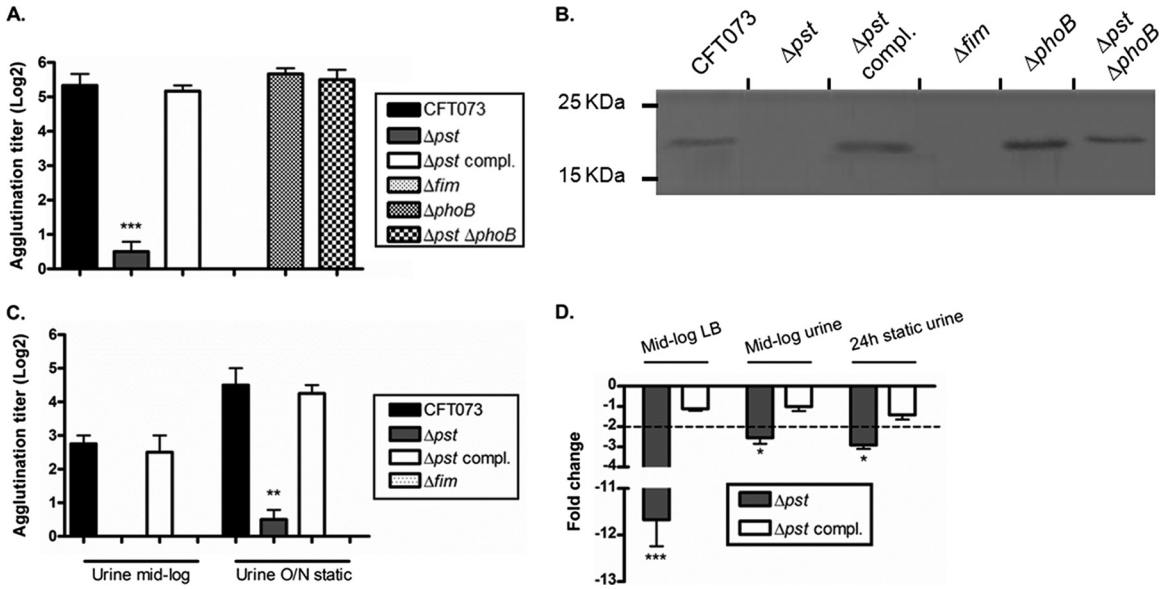

FIG 2 Production and transcription of type 1 fimbriae by uropathogenic *E. coli* CFT073 and derivative strains. (A) Production of type 1 fimbriae in strains cultured to the mid-log phase of growth in LB broth. The Δ*fim* strain was used as a negative control and showed no agglutination. (B) Western blot of fimbrial extracts of strains cultured to the mid-log phase of growth in LB broth. (C) Production of type 1 fimbriae in strains cultured in human urine. (D) Expression of *fimA* in Δ*pst* and Δ*pst* complemented strains compared to the WT CFT073 strain. The dashed line corresponds to the cutoff a significant difference in expression. Results are the mean values and standard deviations for four biological experiments. Statistical significance was calculated by the Student *t* test: ∗, $P < 0.05$; ∗∗, $P < 0.005$; ∗∗∗, $P < 0.0001$.

though it was still significantly ($P = 0.02$) less competitive (about a 3-fold difference compared to the WT) in the kidneys.

To determine whether the outcompeted phenotype observed *in vivo* for the *pst* mutant was due to different growth kinetics, an *in vitro* competition assay was performed in human urine, which represents an *ex vivo* condition that may reflect nutrient availability and environmental conditions encountered in the bladder. Although the *pst* mutant was outcompeted 2.4-fold at 6 h postinoculation, there was no significant difference at 24- and 48 h (see Fig. S1 in the supplemental material). Hence, the outcompetition of the *pst* mutant was not the consequence of a growth defect.

Since inactivation of the *pst* system constitutively activates the PhoBR two-component regulatory system, we determined whether the constitutive activation of *phoB* was responsible for the attenuated virulence of the *pst* mutant. To do so, coinfection between the *phoB* mutant and the WT CFT073 Δ*lac* strain or the double *pst phoB* mutant and the WT CFT073 Δ*lac* strain was performed. The *phoB* knockout mutant colonized the urinary tract as well as the WT strain (Fig. 1B). Similarly, the double mutant strain was as virulent as the WT strain (Fig. 1B). This phenotype was expected because deletion of *phoB* in the *pst* mutant abrogated the induction of the Pho regulon induced by deletion of the *pst* system (7). Thereby, these results show that inappropriate and constitutive induction of the Pho regulon has a deleterious effect on pathogenicity.

Production and transcription of type 1 fimbriae is reduced in the *pst* mutant. We previously demonstrated that deletion of the Pst system inhibited production of type 1 fimbriae by APEC strain χ7122 (7, 20). Since type 1 fimbriae contributed to UPEC pathogenicity (34, 53), we quantified production of type 1 fimbriae in the UPEC CFT073 strain and its derivative mutants. At the mid-log phase of growth in LB broth (OD 0.6), the *pst* mutant was unable to agglutinate yeast, while the WT and complemented strains, respectively, agglutinated yeast at the same titer (Fig. 2A). As expected, expression of type 1 fimbriae was restored in the double *pst phoB* mutant (Fig. 2A). To corroborate that production of type 1 fimbriae was impaired in the *pst* mutant, Western blotting against the type 1 fimbrial major subunit FimA was performed. Western blotting confirmed an important reduction of FimA production by the *pst* mutant compared to those by WT CFT073, the complemented strain, and the double Δ*pst* Δ*phoB* strain (Fig. 2B). Production of type 1 fimbriae, by yeast agglutination, was also performed with bacteria cultured in human urine. At the mid-log phase of growth (OD of ~0.2), the *pst* mutant did not agglutinate yeast, whereas the WT and complemented strains agglutinated yeast at titer 3 (Fig. 2C). When cultured statically for 24 h at 37°C, the *pst* mutant agglutinated yeast only at the highest-density cell suspension, whereas the WT and complemented strains produced type 1 fimbriae at an agglutination titer of 4.5 (Fig. 2C).

Since deletion of the *pst* system inhibited production of type 1 fimbriae, we investigated if repression of type 1 fimbriae occurs at the transcriptional or posttranscriptional level. To test this, expression of the *fimA* gene was evaluated by real-time quantitative reverse transcription-PCR (qRT-PCR). At the mid-log phase of growth in LB broth, transcription of *fimA* was downregulated 11.7- times in the *pst* mutant (Fig. 2D). When bacteria were grown in human urine to mid-log phase and for 24 h statically, expression of the *fimA* gene was also downregulated 2.6- and 2.9 times, respectively, in the *pst* mutant (Fig. 2D). Although repression of *fimA* in the *pst* mutant grown in human urine was slightly reduced compared with that in LB broth, this reduction correlated to yeast agglutination of cells grown in human urine (Fig. 2C). Again,

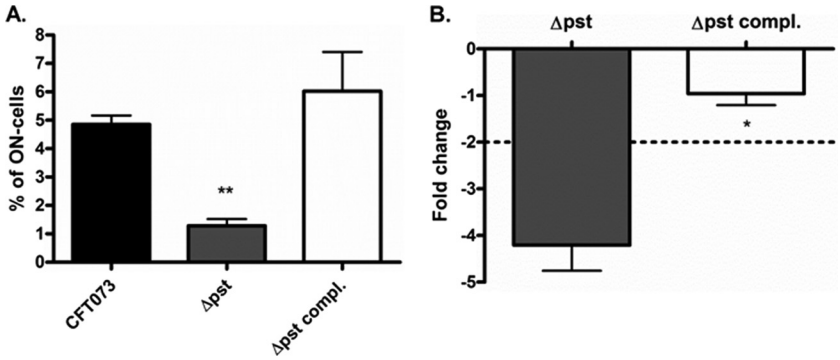

FIG 3 Effect of inactivation of *pst* on orientation of the *fim* promoter (*fimS*) *in vitro*. (A) Quantification of the percentage of cells with *fimS* in the on orientation. (B) On orientation of *fimS* was calculated by qPCR and was compared to that of the WT strain. The dashed line corresponds to the cutoff for a significant difference in expression. Results are the mean values and standard deviations for three biological experiments. Statistical significance was calculated by the Student *t* test: *, $P < 0.05$; ** $P < 0.001$.

complementation of the *pst* mutation restored *fimA* expression to the WT level (Fig. 2D).

Since constitutive induction of the Pho regulon, by inactivation of the *pst* system, repressed expression of *fim*, we tested whether physiological activation of the Pho regulon had the same effect. To do so, bacteria were grown in low-phosphate (LP) medium and expression of *fim* was quantified by qRT-PCR. As for the *pst* mutant, physiological induction of the Pho regulon repressed expression of *fimA*, since no difference was observed between the WT and the *pst* mutant when grown in LP medium (see Fig. S2A in the supplemental material). Furthermore, expression of *fimA* was decreased 14.0-fold in the WT strain cultured in LP compared to that following culture in high-phosphate (HP) medium (see Fig. S2B in the supplemental material). This is similar to what was observed between the *pst* mutant and the WT strain grown in LB medium (Fig. 2D). These results indicate that induction of the Pho regulon, physiologically or by inactivation of *pst*, alters expression of type 1 fimbriae at the transcriptional level.

Hence, under certain growth conditions, the Δ*pst* mutant was impaired in its capacity to produce type 1 fimbriae. This suggests that the attenuation observed in the *pst* mutant could be due, at least in part, to a reduced production of type 1 fimbriae during infection.

Inactivation of the Pst system favors the off orientation of the *fim* promoter. Since expression of the type 1 fimbriae is downregulated in the *pst* mutant, we asked whether its downregulation correlated with the off position of the phase-variable promoter (*fimS*). The orientation of *fimS* was evaluated in strains grown under agitation to mid-log phase in LB broth. Using the classical procedure described by Stentebjerg-Olesen et al. (72), only 1.3% of the *pst* mutant population were in the on position, whereas 4.9% and 6.0% of WT and *pst*-complemented cells were in the on position, respectively (Fig. 3A).

To confirm differences in orientation of the *fimS* switch, we also performed quantitative PCR (qPCR). When the on positions of different strains cultured to mid-log phase in LB broth were compared, the quantity of cells in the on orientation for the *pst* mutant was 4.2-fold lower than that for the WT strain (Fig. 3B), which is similar to results obtained by HinfI digestion of PCR products (3.8-fold) (Fig. 3A). Furthermore, complementation of the *pst* mutant restored the on orientation to the parent level (Fig. 3B). Similar results were obtained for strains cultured in human urine (data not shown). Thereby, in the *pst* mutant, the preferential orientation of the *fim* promoter in the off position in both LB medium and human urine correlated with the decrease in *fim* expression observed.

Expression of the *fimS* recombinases is decreased in the Pst mutant. To correlate the *fimS* off position in the *pst* mutant, expression of the *fimB* and *fimE* recombinases was analyzed by qRT-PCR. Thus, at the mid-log phase of growth in LB broth, *fimB* transcription was downregulated 2.6-fold in the *pst* mutant (Fig. 4A). Expression of *fimB* was restored in the complemented strain. However, no difference was observed for the *fimE* gene (Fig. 4A). In addition to *fimB* and *fimE*, the CFT073 strain encodes the *fim*-like recombinases IpuA and IpbA. Similarly to *fimB*, these two *fim*-like recombinases were downregulated in the *pst* mutant (Fig. 4B). Furthermore, expression of *fimB*, *fimE*, and *fimA* and orientation of *fimS* are known to be controlled by multiple regulators, e.g., *crp*, *hns*, *cyaA*, *rcsB*, *qseB*, *papB*, *nanR*, *ihf*, *lrp*, *nagC*, and *focB*. To determine whether these genes may influence expression of type 1 fimbriae in the *pst* mutant, expression of these regulators was quantified by qRT-PCR. However, none of these regulators was differentially expressed in the *pst* mutant (data not shown).

Because FimB, IpuA, and IpbA promote inversion of *fimS* to the on orientation and therefore positively modulate *fim* expression (10, 40), the off position of *fimS* and the concomitant decreased expression of type 1 fimbriae in the *pst* mutant could be due to the cumulative reduced expression of the *fimB*, *ipuA*, and *ipbA* recombinase-encoding genes.

Inactivation of the Pst system reduces *fimA* expression *in vivo*. To determine whether type 1 fimbrial expression is also downregulated *in vivo*, a time course of single-strain infection in the murine UTI model was performed, since the *pst* mutant was severely attenuated during competitive infection (Fig. 1A). Prior to mouse inoculation, expression of type 1 fimbriae was analyzed by yeast agglutination assay. As shown in Fig. 5A, following overnight growth in LB broth, the *pst* mutant produced type 1 fimbriae as well as the WT strain. At 6 h p.i., the *pst* mutant colonized the mouse urinary tract as well as the WT CFT073 strain (Fig. 5B). However, transcription of *fimA* in the *pst* mutant was downregulated 2.3-fold in the bladder (Fig. 5C). In contrast, at 24 and 48 h p.i., the *pst* mutant was attenuated 28 and 158 times in bladder, respectively (Fig. 5A). Likewise, *fimA* transcription was down-

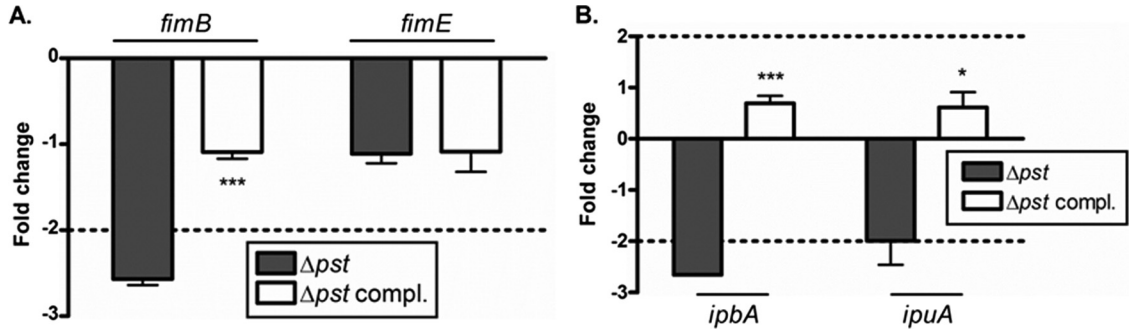

FIG 4 Effect of inactivation of *pst* on *fimS* regulators. Expression of the *fimB*- and *fimE*-encoded recombinases (A) or *fim* recombinase-like *ipbA*- and *ipuA*-encoded recombinases (B) compared to that for the WT strain. The dashed line corresponds to the cutoff for a significant difference in expression. Results are the mean values and standard deviations for three biological experiments. Statistical significance was calculated by the Student *t* test: *, $P < 0.05$; ***, $P < 0.0001$.

regulated 5.5- and 44.0-fold in the bladder at 24 and 48 h, respectively (Fig. 5B). Overall, although production of type 1 fimbriae is similar between the *pst* mutant and the WT strain prior to mouse inoculation, *in vivo*, expression of *fimA* was decreased at all times postinfection in the *pst* mutant, and differences were more marked at later time points.

Orientation of the *fim* promoter (*fimS*) was analyzed to correlate with expression of *fimA* in the *pst* mutant in infected bladder. At all time points, *fimS* was more frequently in the off position in the *pst* mutant than in the WT strain. This was most marked at 48 h, when the on position of *fimS* was decreased 56.1-fold (Fig. 5D), which correlates with the concomitant downregulation of *fimA* observed at that time (Fig. 5C). Expression of the *fim* recombinase genes *fimB* and *fimE* was determined at 6, 24, and 48 h p.i. in the bladder. Expression of *fimE* was increased in the *pst* mutant at each time point p.i., and this upregulation was more marked at later times (Fig. 5E). Thus, compared to that in the WT strain, expression of *fimE* in the *pst* mutant was increased 2.2- and 3.9-fold at 24 and 48 h p.i., respectively (Fig. 5E).

Taken together, these results confirm that inactivation of *pst* represses expression of type 1 fimbriae and could explain the attenuation of the *pst* mutant.

Attenuation of the *pst* mutant is due mainly to the downregulation of type 1 fimbriae. Since we previously demonstrated that decreased virulence of *pst* mutants in ExPEC strains may be the consequence of surface perturbations (7, 20, 42–44), we compared membrane integrity of the UPEC CFT073 *pst* mutant. Although the *pst* mutant demonstrated intermediate resistance to 90% human serum up to 2 h postincubation (Fig. 6A), the *pst* mutant was as resistant as the WT strain to hydrogen peroxide, polymixin B, and vancomycin (Fig. 6B, C, And D, respectively), since no statistical difference was observed.

To test whether attenuation of the *pst* mutant is mainly the consequence of downregulation of type 1 fimbriae, we constitutively induced expression of type 1 fimbriae by introducing point mutations into the right inverted repeat of *fimS*, which locked the promoter switch in the on position (L-ON). We then compared the capacity of the WT and Δ*pst fim* L-ON derivative strains to colonize the mouse urinary tract. At 48 h p.i., no significant difference in colonization of the bladder was observed between the CFT073 *fim* L-ON and Δ*pst fim* L-ON mutant strains, whereas the Δ*pst fim* L-ON strain was attenuated 2.5 times in kidneys (Fig. 6E). By comparing the colonization of the mouse urinary tract between the *pst* (Fig. 5B) and *pst fim* L-ON mutants (Fig. 6E), we observed that the *fim* L-ON strain colonized the bladder and kidneys 1,384 and 3 times more, respectively, than the *pst* mutant. Thereby, these results show the major contribution of the downregulation of type 1 fimbriae in attenuation of the *pst* mutant during infection, which is more marked in bladder, where type 1 fimbriae are required.

Concentration of ppGpp is diminished in the *pst* mutant. The alarmone ppGpp is a second messenger produced by nutritional starvation and initiates global physiological adaptation (21). Its synthesis relies on RelA and SpoT, which encode the GDP pyrophosphokinase/GTP pyrophosphokinase and guanosine 3′-diphosphate 5′-triphosphate 3′-diphosphatase, respectively. In addition to its role in adaptation to starvation, ppGpp negatively regulates expression of type 1 fimbriae. Indeed, accumulation of ppGpp induces transcription of *fimB* and thus *fimA* (1, 2). Since it has been previously observed that mutations in the *pst* system decreased the concentration of ppGpp (70, 71), we hypothesized that the downregulation of *fimB* and the reduced production of type 1 fimbriae *in vitro* may be mediated by a decrease in the ppGpp concentration in the *pst* mutant.

At the mid-log phase of growth in LB medium, the mean intracellular pool of ppGpp in the WT CFT073 strain was 45.65 nM and that of the *pst* mutant was 15.02 nM, which represents a decrease of 3-fold (Fig. 7). Although complementation did not completely restore the concentration of ppGpp to the WT level, no statistical difference was observed between the complemented strain and the WT parent (Fig. 7). These results demonstrate that during mid-log growth, the *pst* mutant produces less ppGpp than the WT strain, and they could explain, at least in part, the decreased expression of *fimB* and reduced expression of type 1 fimbriae in the *pst* mutant.

Deletion of *pst* genes induces expression of F1C fimbriae. It has been observed that deletion of the *fim* operon resulted in enhanced expression of other types of fimbriae (67). In this manner, since the Δ*pst* strain colonized the bladder as well as the WT strain up to 6 h p.i., production of other adhesins could compensate for the loss of type 1 fimbriae and then mediate adherence to epithelial cells.

To test this possibility, adhesion assays using 5637 human bladder epithelial cells (ATCC HTB-9) were performed. The Δ*pst* mutant adhered to human bladder cells as well as the WT strain (Fig. 8A and B). Interestingly, the CFT073 Δ*fim* strain also ad-

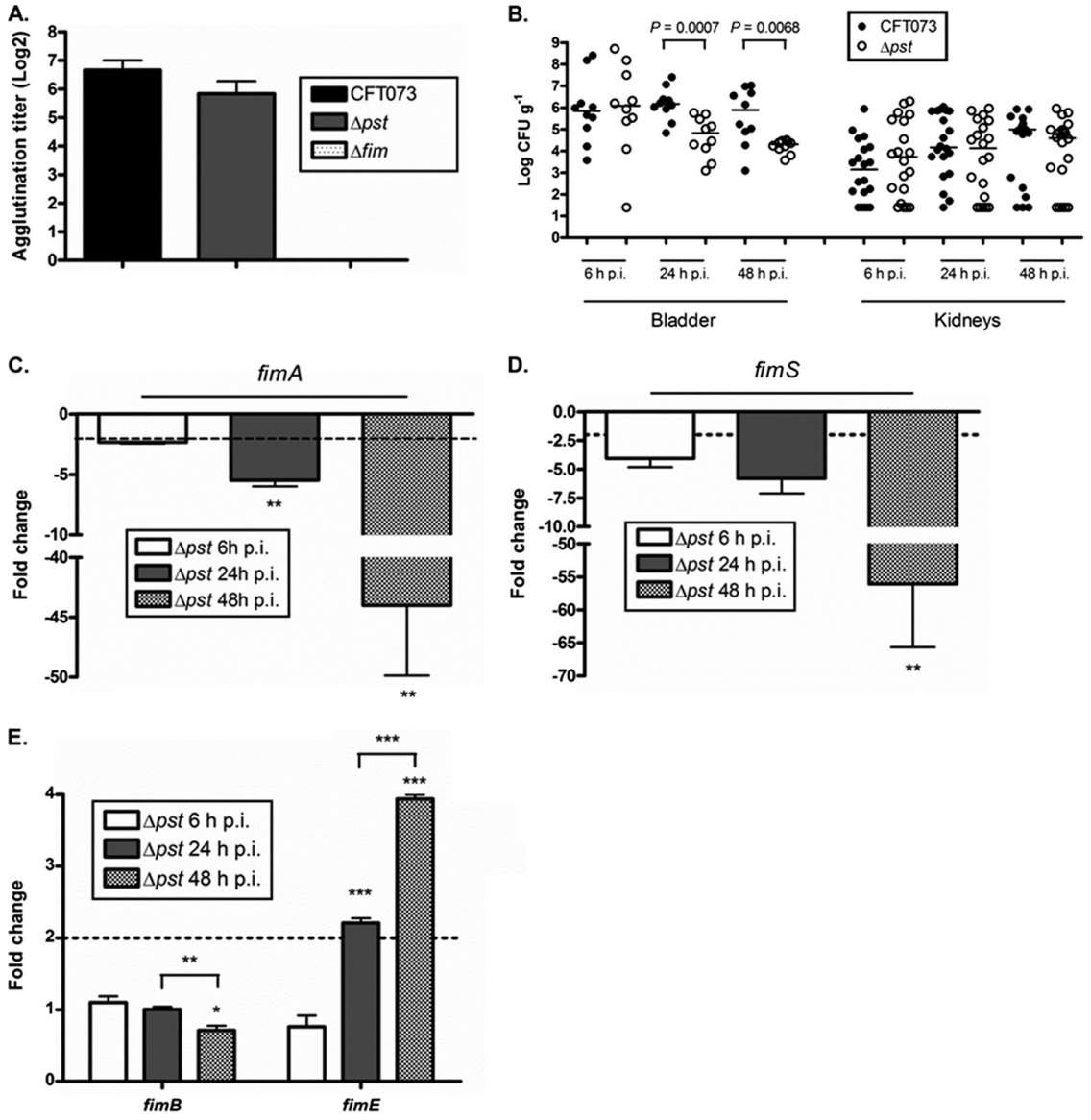

FIG 5 Inactivation of *pst* reduces colonization and results in differential expression of *fim* genes by uropathogenic *E. coli* CFT073 in the mouse bladder. (A) Production of type 1 fimbriae *in vitro* prior to inoculation. The Δ*fim* mutant was used as a negative control since it did not mediate yeast agglutination. (B) Time course infection of CBA/J mice. Bacterial numbers are presented as the $\log_{10}$ CFU g^{-1}. Each data point represents a sample from an individual mouse, and horizontal bars indicate the median values. Each kidney was sampled separately. (C) Expression of *fimA* in infected bladders for the *pst* mutant compared to that for the WT strain. (D) Orientation of the *fim* promoter (*fimS*) in infected bladders for the *pst* mutant compared to that for the WT strain. (E) Transcription of the *fim* recombinase genes *fimB* and *fimE* in infected bladders for the *pst* mutant compared to that for the WT strain. The dashed line corresponds to the cutoff for a significant difference in expression. All results shown are the mean values and standard deviations for each bladder infected with the WT and the *pst* mutant strain. Statistical significance was calculated by the Student *t* test (A, C, D, and E) (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$) or by the Mann-Whitney test (B).

hered as well as the WT strain (Fig. 8A), which supports the hypothesis that other fimbriae or adhesins are produced that compensate for the loss of the type 1 fimbriae in the *pst* mutant. Locking on the *fim* promoter increased adherence of the WT and the *pst* mutant to 42.94 and 48.17%, respectively (Fig. 8A). Because type 1 fimbriae recognize mannosylated residues on host epithelial cells, addition of soluble mannose to culture medium will inhibit type 1 fimbria-dependent adhesion to 5637 cells. Addition of 1.5% α-D-mannopyranose decreased adherence of all strains, except for the Δ*pst* and Δ*fim* mutants, to 5637 bladder cells, to approximately 6.5% (Fig. 8A and B). However, addition of α-D-mannopyranose did not reduce adherence of either the *pst* mutant, or the Δ*fim* strain (Fig. 8A and B). Not surprisingly, deletion of *phoB* in the *pst* mutant restored mannose-sensitive adhesion to bladder cells (Fig. 8A).

Since the *pst* and *fim* mutants adhered to bladder cells independently of type 1 fimbriae, the presence of fimbrial structures at the cell surface of the *pst* and *fim* mutant strains was analyzed by electron microscopy. Indeed, fimbriae were observed on their cell surface (Fig. 8C). This confirms that adherence of the *pst* mutant to bladder cells is independent of type 1 fimbriae and is mediated by other adhesins present at the cell surface.

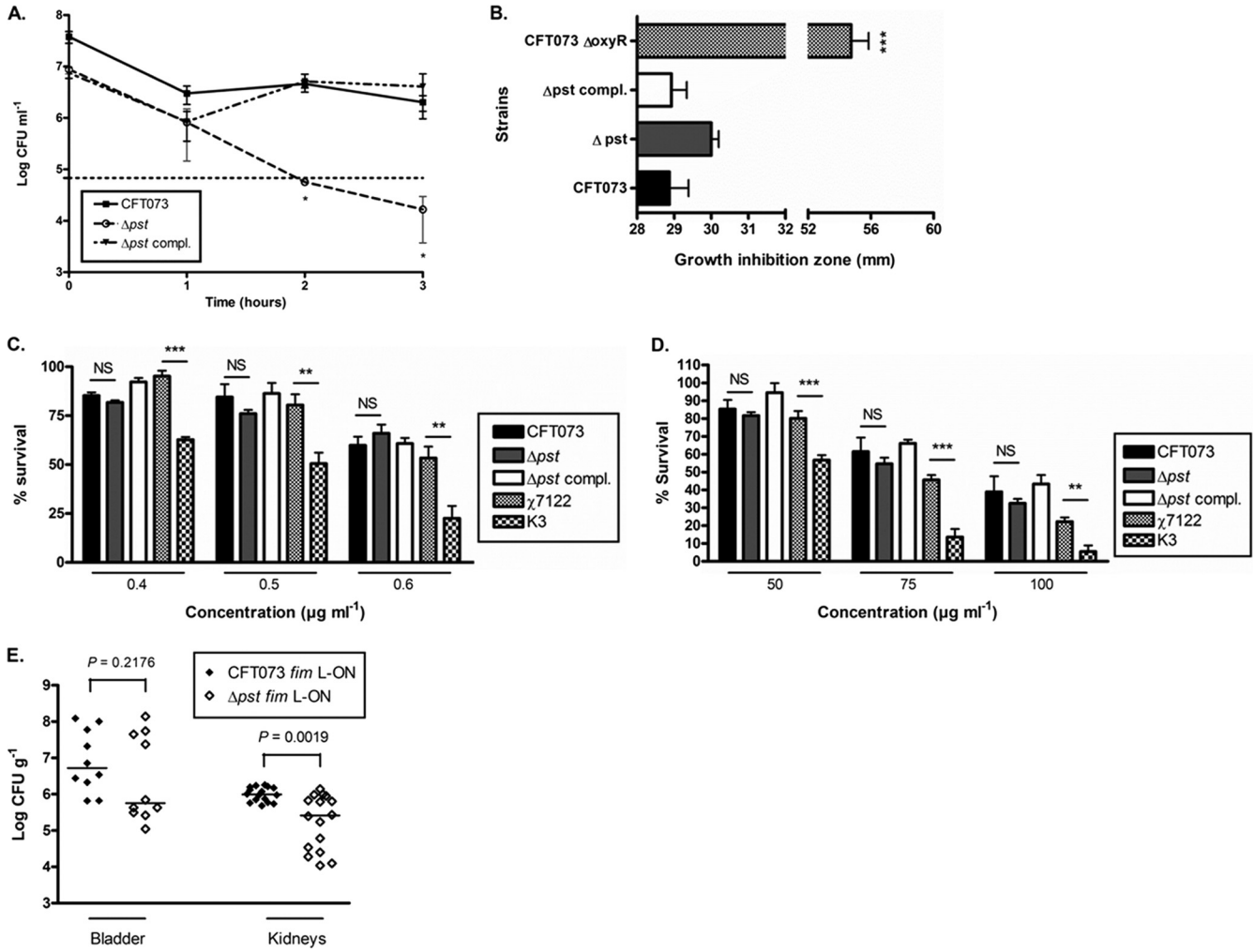

FIG 6 Altered expression of type 1 fimbriae is linked to attenuation of the *pst* mutant. (A) Resistance to 90% human serum. MG1655 was used as negative control and was sensitive at the 1-h time point. The dashed line corresponds to the intermediate resistance limit. (B) Resistance to hydrogen peroxide. The CFT073 Δ*oxyR* strain was used as negative control. (C) Resistance to polymixin B. The χ7122 and K3 strains were used as positive and negative controls, respectively. (D) Resistance to vancomycin. The χ7122 and K3 strains were used as positive and negative controls, respectively. (E) Monoinfection of CBA/J mice with CFT073 and Δ*pst fim* L-ON derivative strains. Bacterial numbers are expressed as $\log_{10}$ CFU g^{-1}. Each data point represents a sample from an individual mouse, and horizontal bars indicate the median values. Results from panels A to D are the mean values and standard deviations for four biological experiments. Statistical significance was calculated by Student's *t* test (A to D) (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$) or by the Mann-Whitney test (E).

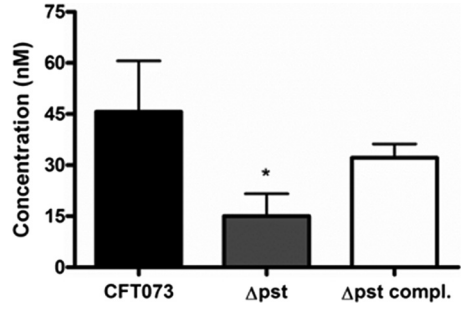

FIG 7 Production of ppGpp in the *pst* mutant. Production of ppGpp in strains grown to the mid-log phase of growth in LB medium. All results shown are the mean values and standard deviations for three biological experiments. Statistical difference was calculated by one-way ANOVA with Bonferroni's multiple-comparison test.

In UPEC strain CFT073, it was previously shown that when type 1 fimbriae were phase locked off, Pap fimbrial gene expression increased (67). To determine if Pap fimbriae were upregulated in the *pst* mutant, mannose-resistant hemagglutination (MRHA) of human erythrocytes onto strains cultured in LB medium was performed. As shown in Fig. 8D, no differences in MRHA between the WT and the *pst* mutant were observed, which suggests that Pap fimbriae were similarly expressed between these strains. To confirm the MRHA results, expression of the genes encoding the Pap fimbrial major subunits, *papA* and *papA_2*, following adherence to 5637 bladder cells, which may reflect adhesion to bladder cells observed *in vivo*, was determined. Similarly to findings of the MRHA experiments, no difference was observed between strains (Fig. 8E). These results demonstrate that the mannose-resistant adherence of the *pst* mutant to bladder epithelial cells is not attributed to increased production of Pap fimbriae. It

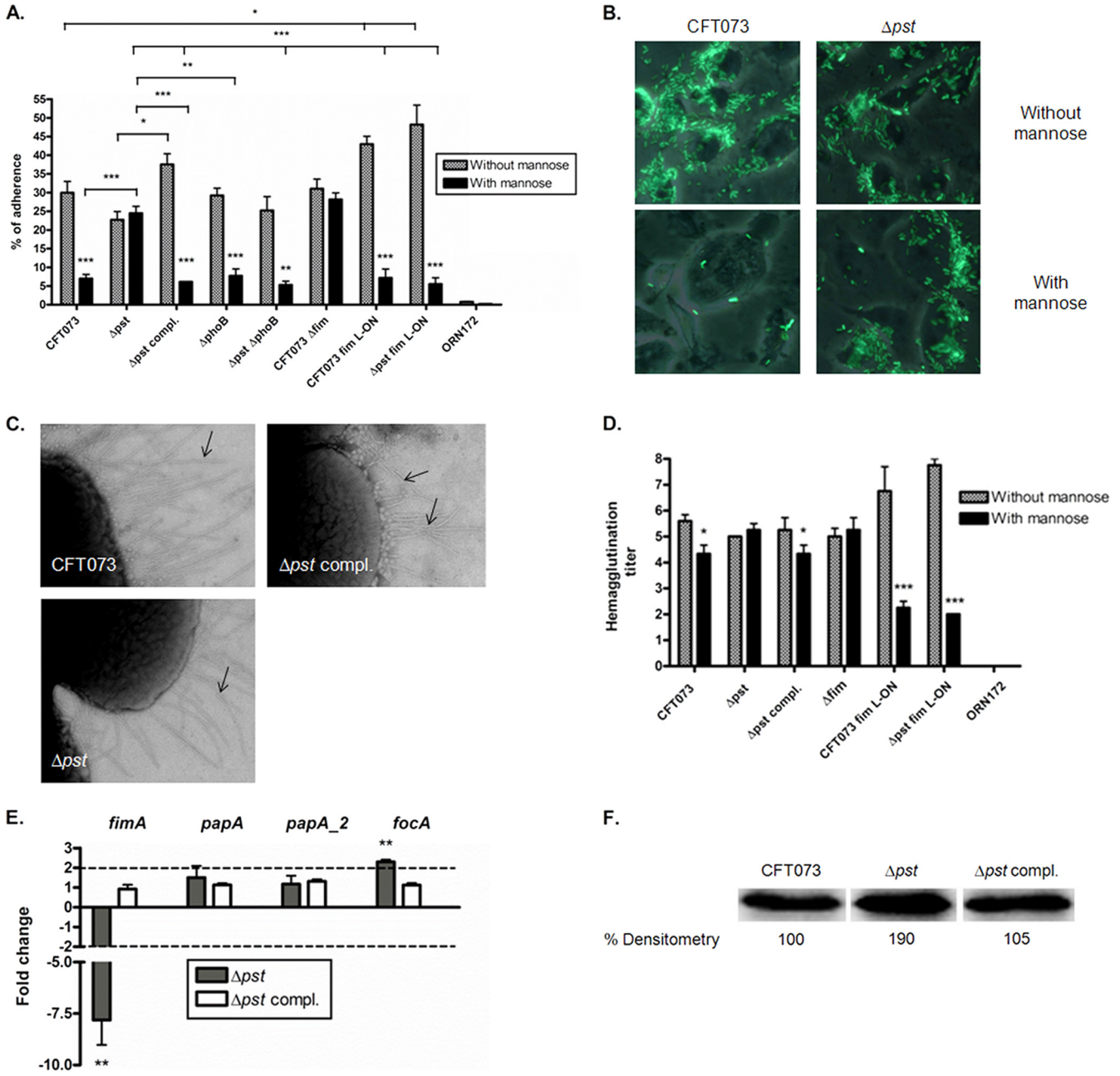

FIG 8 Effect of inactivation of *pst* and production of type 1 fimbriae on adherence of uropathogenic *E. coli* CFT073 to human bladder epithelial cells *in vitro*. (A) Adherence of strain CFT073 and its derivatives to human 5637 bladder epithelial cells in the presence or absence of 1.5% α-D-mannopyranose. The *fim*-negative *E. coli* K-12 strain ORN172 was used as a negative control. (B) Fluorescence microscopy of infected 5637 cells with CFT073-GFP and the Δ*pstSCA*-GFP strain (magnification, ×200). Images are a representative field. (C) Electron microscopy of CFT073 and the Δ*pstSCA* and Δ*fim* strains, respectively, at magnification ×12,000. Images show a typical field of view of bacteria. Arrows show fimbriae on cell surfaces. (D) Production of Pap fimbriae by hemagglutination assay. (E) Transcription of *fimA*, *papA* major subunits (*papA* and *papA_2*) and *focA* (F1C fimbria-encoding) from bacteria adhered to 5637 bladder cells compared to results for the WT CFT073 strain. The dashed line corresponds to the cutoff for a significant difference in expression. (F) Western blot of fimbrial extracts using F1C-specific (anti-$F165_2$) antiserum. Densitometry of the band from the WT CFT073 strain was considered to be 100%. Bands and densitometry are from a representative gel. All results shown are the mean values and standard deviations for four biological experiments. Statistical significance was calculated by the Student *t* test (A, D, E, and F): *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

was also previously shown that expression of F1C fimbriae increased considerably in a strain lacking the *fim* and *pap* gene clusters (67). Thereby, we investigated by qRT-PCR and Western blotting whether expression of F1C fimbriae was increased in this background. As shown in Fig. 8E and F, F1C fimbriae were upregulated 2-fold in the *pst* mutant. Therefore, upregulation of F1C fimbriae in the *pst* mutant could contribute to *in vitro* adherence of the *pst* mutant to bladder cells.

Deletion of *pst* genes reduces invasion of 5637 human bladder cells *in vitro*. It has previously been shown that some UPEC

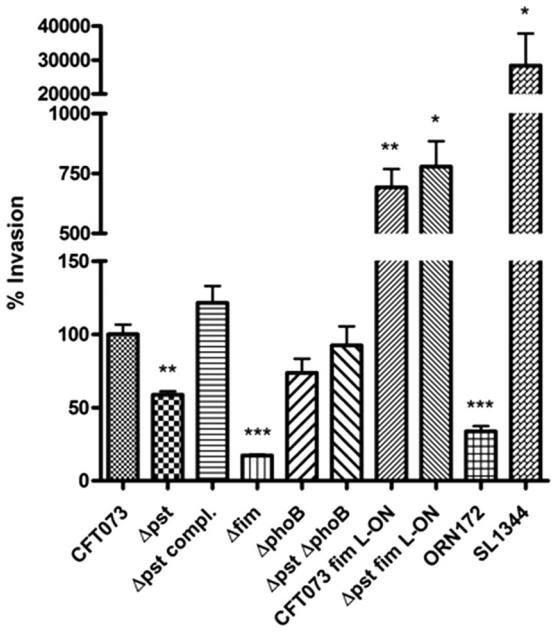

FIG 9 Invasion assay using 5637 bladder cells with UPEC CFT073 and derivative strains. The *Salmonella enterica* Typhimurium strain SL1344 was used as a positive control for invasion. All results shown are the mean values and standard deviations for four biological experiments. Statistical significance was calculated by the Student *t* test: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

strains can invade bladder epithelial cells (38) and that epithelial cell invasion is mediated by type 1 fimbriae (46, 51). We therefore hypothesized that the *pst* mutant may demonstrate reduced cell invasion. Indeed, cell invasion by the *pst* mutant was significantly reduced, to 59% of the invasive capacity of the WT strain (Fig. 9). Similarly, for the Δ*fim* strain, invasion was decreased to 17.3% of the WT level despite the fact that it adhered as well as the WT strain to bladder cells. Deletion of *phoB* in the *pst* mutant restored the invasion capacity of the Δ*pst* strain. Finally, invasion of the CFT073 *fim* L-ON and Δ*pst fim* L-ON derivative strains increased to 693 and 779%, respectively, confirming the role of type 1 fimbriae in bladder epithelial cell invasion.

Taken together, these results demonstrate that although expression of the *fim* operon is downregulated in the *pst* mutant, expression of other adhesins can mediate adherence to bladder cells. However, decreased expression of type 1 fimbriae results in a reduced invasive capacity of the *pst* mutant and could explain the decreased colonization of the bladder.

DISCUSSION

In this study, we determined that the attenuation of the UPEC CFT073 *pst* mutant strain could be attributed primarily to the downregulation of type 1 fimbriae. We showed that the deletion of the Pst system repressed transcription and production of type 1 fimbriae both *in vitro* and *in vivo*. Since type 1 fimbriae are essential for bladder colonization and invasion (34, 53), downregulation of the *fim* genes could explain the attenuated virulence of the *pst* mutant. Indeed, in the bladder, *fimA* expression was progressively reduced during infection.

We observed that the *fim* promoter had an increased bias for the off position in the *pst* mutant *in vitro* and *in vivo*. In LB medium, the recombinase-encoding genes *fimB*, *ipuA*, and *ipbA* were downregulated in the *pst* mutant, whereas *fimE* was not differentially expressed. Since the recombinases FimB, IpuA, and IpbA promote expression of the *fim* operon by orienting the *fimS* promoter to the on position (10, 26, 40), their downregulation correlates with the downregulation of the *fim* operon and the increased orientation of *fimS* to the off position in the *pst* mutant. We hypothesized that the differential expression of *fimB* in the *pst* mutant was the major factor leading to decreased expression of type 1 fimbriae in the *pst* mutant. Indeed, overexpression of *fimB* in the *pst* mutant restored production of type 1 fimbriae to levels similar to those of the *fim* locked-on strain (data not shown).

In infected bladders, the inverse phenomenon regarding *fimB* and *fimE* gene expression was observed, i.e., *fimB* was not differentially expressed, whereas transcription of *fimE* was increased, in the *pst* mutant. The differential expression of *fimB* and *fimE in vitro* and *in vivo* could be due to differences in culture conditions, i.e., LB medium versus bladder. It was previously observed that human urine decreased expression of *fimB* and increased that of *fimE* (63). Furthermore, Sohanpal et al. (69) have demonstrated that the presence of *N*-acetyl-β-glucosaminidase, a lysosomal enzyme found in high concentrations in the upper urinary tract, repressed expression of *fimB*. By comparing expression of *fimB* and *fimE* in bacteria grown in either human urine or LB medium, we observed that in the WT and Δ*pst* strains, expression of *fimB* is slightly increased in human urine (see Fig. S3 in the supplemental material). Furthermore, in accordance with the work of Schwan et al. (63), expression of *fimE* was increased 8.63-fold in the WT strain cultured in human urine (see Fig. S3 in the supplemental material). Furthermore, disruption of the *pst* system enhanced this upregulation, since *fimE* was increased 26.3-fold in the Δ*pst* strain. Since FimE reduces expression of *fim* by promoting the off position (26, 40), its induction, in infected bladder may also direct the orientation of the *fim* promoter to the off position, leading to downregulation of type 1 fimbriae in the *pst* mutant.

Constitutive expression of the *fim* operon in the *pst* mutant restored production of fimbriae at the cell surface and colonization of the bladder to the WT level. This observation is not in agreement with the hypothesis that attenuation of the *pst* mutant is the consequence of pleiotropic effects and membrane perturbation described elsewhere (7, 20, 42–44). In contrast to *pst* mutants of other ExPEC strains, the *pst* mutant of UPEC CFT073 did not demonstrate marked phenotypes associated with membrane perturbation. Indeed, the CFT073 *pst* mutant showed intermediate resistance to 90% human serum up to 2 h postincubation and resisted oxidative stress, polymixin B, and vancomycin to levels that were not significantly different from those for the WT parent. Therefore, in contrast to the APEC strain, the CFT073 *pst* mutant demonstrates fewer pleiotropic effects. As such, the effects of the *pst* mutation on downregulation of type 1 fimbriae in strain CFT073 are the major cause of the reduced colonization of the urinary tract. However, because constitutive activation of the Pho regulon through inactivation of Pst results in a cascade of regulatory changes, it is likely that a number of regulatory changes which may or may not lead to a reduction in type 1 fimbrial expression contribute to reduced virulence.

Production of ppGpp could be one of the mechanisms by which the Pho regulon influences expression of type 1 fimbriae. Indeed, it has been observed that accumulation of ppGpp activates transcription of *fimB* and concomitantly *fimA* (1, 2). In our current study, at the mid-log phase of growth, the *pst* mutant was

shown to produce 3-fold less ppGpp than the wild-type parent strain, CFT073 (Fig. 7). Since a decreased concentration of ppGpp alters the transcription of *fimB* and the *fim* operon (1, 2), we suggest that the downregulation of *fimB* and consequently the *fim* operon could be due to the decreased concentration of ppGpp in the *pst* mutant.

In either competitive or single-strain infections, the *pst* mutant was attenuated in the murine UTI model. However, the *pst* mutant was less attenuated in single-strain infections. Since adhesion to bladder cells, via type 1 fimbriae, induces inflammation and recruitment of neutrophils to bladder (53), it is possible that in the competitive infection, the innate immune response is accentuated due to the presence of the WT strain. This increased induction could result in the loss of fitness of the *pst* mutant in the competitive infection and may explain its pronounced attenuation at 48 h p.i. in both bladder and kidneys.

In vitro, the *pst* mutant adhered to human bladder cells as well as the WT strain, which correlates with the colonization of the mouse bladder up to 6 h p.i. However, compared to the WT strain, this adhesion was mannose resistant. Electron microcopy demonstrated fimbriae at the cell surface of the *pst* mutant, indicating that other adhesins are expressed at the bacterial cell surface and could mediate adherence to bladder epithelial cells. Interestingly, the *fim* mutant strain also adhered to bladder cells as well as the WT, in a mannose-resistant manner, and produced fimbriae at its cell surface. In addition, the *pst* mutant had the same colonization kinetics as the Δ*fim* strain in the murine UTI model (see Fig. S4 in the supplemental material), where bladder and kidney colonization by the Δ*fim* strain is in agreement with the study of Gunther et al. (30). UPEC strain CFT073 contains genes predicted to encode 12 distinct fimbriae and many afimbrial adhesins (80). Since one type of fimbria is predominantly expressed at a time (54, 67), they are coordinately expressed and are subjected to a regulatory cross talk (82). Thereby, it is not surprising to note the presence of other fimbriae at the cell surface of the *pst* and *fim* mutants. In this study, we observed that F1C fimbriae were increased in the *pst* mutant. Since F1C fimbriae mediate adhesion to bladder and kidney epithelial cells (78), the observed adhesion of the *pst* mutant could be attributed to F1C fimbriae. However, further experiments are required to determine the precise contribution of F1C fimbriae in the *pst* mutant.

In addition to mediating adherence, type 1 fimbriae are also involved in invasion of bladder epithelial cells (34, 38). Although the *pst* mutant adhered to bladder cells as well as the WT strain, its invasion capability was reduced. This invasion defect is directly linked to the downregulation of the *fim* genes, since constitutive expression of type 1 fimbriae in the *pst* mutant considerably increased invasion. It has been postulated that invasion of bladder cells contributes to UPEC pathogenesis since invasion can provide refuge from both innate and adaptive host immune defenses (25, 52). In this manner, intracellular UPEC can serve as a source for recurrent UTIs (34, 38). Thereby, the decreased invasion rate of the *pst* mutant could explain, at least in part, its attenuation of virulence. Reduced bladder colonization observed in the *pst* mutant at 48 h p.i. could also be attributed to its invasion defect.

In previous reports, inactivation of different TCSs in *E. coli* reduced cell adherence and inhibited the expression of different types of fimbriae (37, 41, 56, 64). In our case, by contrast, we have demonstrated here that induction of the PhoBR TCS, by either deletion of the *pst* system or phosphate starvation, results in repression of type 1 fimbriae. Because the increase in antimicrobial resistance of bacterial pathogens is a major problem, it is important to develop novel preventive and therapeutic strategies. Since TCSs are required for the adaptive response to environmental stimuli and they are absent from humans and other animals, they represent choice targets for therapeutic strategies. Indeed, a number of chemical inhibitors of TCSs have been identified by screening libraries of synthetic compounds (74, 83). Furthermore, small chemical molecules targeting different virulence factors have been shown to be useful in preventing infection from different pathogens (13, 14, 27, 59). In this manner, small molecules targeting the Pst system or PhoBR could be considered as therapeutic agents to constitutively activate the Pho regulon and potentially prevent such pathogens from causing infections.

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