

PhoU enhances the ability of extraintestinal pathogenic *Escherichia coli* strain CFT073 to colonize the murine urinary tract

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The *phoU* gene is the last cistron in the *pstSCAB-phoU* operon and functions as a negative regulator of the Pho regulon. The authors previously identified a *phoU* mutant of extraintestinal pathogenic *Escherichia coli* strain CFT073 and demonstrated that this mutant was attenuated for survival in the murine model of ascending urinary tract infection. It is hypothesized that the PhoU protein might serve as a urovirulence factor by indirectly affecting the expression of virulence-related genes. In this study, the *phoU* mutant was further characterized and PhoU was confirmed as a virulence factor. Western blot analysis demonstrated that insertion of the transposon in the *phoU* gene disrupted the expression of PhoU. The *phoU* mutant had derepressed alkaline phosphatase activity under phosphate-excess and -limiting conditions. In single-challenge murine ascending urinary tract infection experiments, quantitative cultures of urine, bladder and kidney revealed no significant differences between the *phoU* mutant strain and the wild-type strain CFT073. However, in competitive colonization experiments, the *phoU* mutant strain was significantly out-competed by the wild-type strain in the kidneys and urine and recovered in lower amount in the bladder. Complementation of the *phoU* mutant with a plasmid containing the wild-type *phoU* gene restored the expression of PhoU and alkaline phosphate activity to wild-type levels and no significant difference in colonization was observed between the *phoU* mutant containing the complementing plasmid and wild-type in competitive colonization experiments. In human urine, the *phoU* mutant and wild-type grew comparably when inoculated independently, indicating that the attenuation observed was not due to a general growth defect. However, as observed *in vivo*, the wild-type out-competed the *phoU* mutant in competition growth experiments in human urine. These data indicate that PhoU contributes to efficient colonization of the murine urinary tract and add PhoU to a short list of confirmed urovirulence factors.

Received 20 June 2005

Revised 27 September 2005

Accepted 11 October 2005

INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains cause urinary tract infections (UTIs), neonatal meningitis and sepsis. UTIs are among the most common bacterial infections; in the USA they account annually for 7–8 million visits to physicians, 1–2 million emergency department visits, 100 000–400 000 hospitalizations and costs in excess of 3 billion dollars (Foxman *et al.*, 2000; Litwin *et al.*, 2005). Thus, UTIs represent a significant medical-economic burden to the healthcare system. Most UTIs develop by the ascending route of infection, by which periurethral microorganisms enter through the urethra and progress upward

into the bladder lumen. The bacteria may ascend further via the ureters into the kidneys and even breach the kidney parenchyma to enter the lymphatic system or the bloodstream. Therefore, the manifestations of UTI can range from asymptomatic bacteriuria to urethritis, cystitis, pyelonephritis, bacteraemia and septic shock. *E. coli* is responsible for 70–90% of the seven million cases of acute cystitis and 250 000 cases of pyelonephritis reported annually in the USA.

Certain O:K:H serotypes and virulence factors occur more frequently in urinary isolates than in faecal isolates, suggesting that uropathogenic *E. coli* (UPEC) strains are different from commensal *E. coli*. Recent evidence suggests that similar factors and clones are found among other extraintestinal *E. coli* infections, indicating that ExPEC are indistinguishable from UPEC (Johnson & Russo, 2002). A

Abbreviations: ExPEC, extraintestinal pathogenic *E. coli*; STM, signature-tagged mutagenesis; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

limited number of virulence factors, including adhesins, iron-uptake systems, toxins and capsules, have been implicated as important traits allowing these strains to cause disease. Nonetheless, no single virulence factor or set of factors can uniquely identify UPEC.

Our laboratory studies the ExPEC strain CFT073, a highly virulent strain isolated from the blood of a woman with acute pyelonephritis (Mobley *et al.*, 1990). This strain is considered to be a prototype of the O6 serogroup and its virulence has been reproduced in the well-established CBA mouse model of ascending UTI (Mobley *et al.*, 1990). Potential urovirulence factors that have been identified for CFT073 and other ExPEC strains include type 1 fimbriae, secreted autotransporter toxin, cytotoxic necrotizing factor, iron-transporting outer-membrane protein TonB, osmo-protectant ProP, transcriptional regulator RfaH and DegS (Connell *et al.*, 1996; Bahrani-Mougeot *et al.*, 2002; Guyer *et al.*, 2002; Rippere-Lampe *et al.*, 2001; Torres *et al.*, 2001; Culham *et al.*, 1998; Nagy *et al.*, 2002; Redford *et al.*, 2003). Of these factors, only type 1 fimbriae, TonB, RfaH and DegS have been proven to be required for pathogenesis by inactivation of genes required for their expression and complementation to restore the virulence defect. Recently, the genomic sequence of CFT073 was reported and revealed additional factors that may contribute to the pathogenesis of UTIs, such as additional fimbriae, several autotransporters and a type I RTX-like secretion system (Welch *et al.*, 2002).

Previously, we used signature-tagged mutagenesis (STM) to identify genes that are essential for the survival of CFT073 within the murine urinary tract (Bahrani-Mougeot *et al.*, 2002). Among the genetic loci implicated by this study was the *phoU* gene. The *phoU* gene is the last cistron in the *pstSCAB-phoU* operon, which encodes a phosphate-specific transport system (Pst) that actively transports inorganic phosphate (P_i) into *E. coli* cells (Surin *et al.*, 1985). The Pst system is part of the Pho regulon and is induced under P_i starvation conditions (Wanner, 1996). PstS is the periplasmic P_i -binding protein; PstA and PstC are integral membrane proteins that mediate the translocation of P_i through the inner membrane; and PstB is an ATPase that energizes the transport. PhoU is a peripheral membrane protein and its role in phosphate transport is controversial (Surin *et al.*, 1985; Nakata *et al.*, 1984). The *phoU35* allele, which results in the replacement of alanine-147 with glutamate, has no effect on phosphate uptake. However, a *phoU* deletion mutant has been reported to have reduced phosphate uptake (Muda *et al.*, 1992). In contrast, several *phoU* alleles, including two missense mutations and an insertion, have been reported to lead to increased intracellular accumulation of polyphosphate (Morohoshi *et al.*, 2002). Yet another *phoU* deletion mutant was reported to be unaffected in phosphate transport (Steed & Wanner, 1993). Interestingly, this last mutant had a severe growth defect and it was suggested that spontaneous compensatory mutations in the *pst* operon, *phoB* or *phoR* may have alleviated this defect.

The Pst system also negatively regulates the Pho regulon, a set of genes and operons that are regulated by the concentration of P_i in the medium. Mutations in the *pst* genes, as well as *phoU*, lead to constitutive expression of all Pho genes, such as *phoA*, encoding alkaline phosphatase. However, PhoU does not appear to act directly as a repressor, since overexpression of PhoU under low phosphate does not lead to decreased alkaline phosphatase expression (Muda *et al.*, 1992). The Pho regulon including the *pstSCAB* genes is co-regulated by the PhoR–PhoB two-component regulatory system. Under P_i starvation conditions the sensor kinase PhoR phosphorylates PhoB, and phosphorylated PhoB in turn activates transcription of the Pho regulon genes. It has been suggested that PhoU interacts with components of the PstSCAB transporter under conditions of phosphate excess to form a repressor complex and that this complex acts a negative regulator (Steed & Wanner, 1993). However, direct evidence of such interactions is lacking.

In this study, we further characterize the *phoU* mutant of CFT073 in an effort to determine its effect on the Pho regulon, on growth and on colonization in the murine model of ascending UTI.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain CFT073 was isolated from the blood of an otherwise healthy woman admitted to the University of Maryland Medical Center with the clinical syndrome of acute pyelonephritis. CFT073 is highly virulent in the murine urinary tract and its genome has been sequenced (Welch *et al.*, 2002). Bacteria were stored at -70°C in 50% Luria–Bertani (LB) broth and 50% glycerol and were routinely grown at 37°C in LB broth or on Luria agar supplemented with appropriate antibiotics. Antibiotics were used at the following concentrations: ampicillin, $50\ \mu\text{g ml}^{-1}$; kanamycin, $50\ \mu\text{g ml}^{-1}$; nalidixic acid, $50\ \mu\text{g ml}^{-1}$; and rifampicin, $50\ \mu\text{g ml}^{-1}$.

Recombinant DNA methods. All DNA manipulations were carried out by using standard procedures (Sambrook *et al.*, 1989). The enzymes and chemicals used for DNA manipulation were purchased from Invitrogen and New England Biolabs. DNA fragments used in the cloning procedures and PCR products were isolated from agarose gels with the Qiaquick gel extraction kit (Qiagen). Plasmid DNA from *E. coli* was isolated and purified with a Wizard Plus minipreps DNA purification system (Promega) or a Qiagen plasmid midi kit. Plasmids were introduced into *E. coli* by electroporation or by chemical methods. Primers used in this study were synthesized at the University of Maryland School of Medicine.

Complementation of the *phoU* mutant. The full-length coding region of *phoU* was amplified by PCR using primers Donne 904 (5'-CCC GAC TTC ACC AGT ATT CTC TGG TTA TGT CAG G-3') and Donne 905 (5'-CGG CAC TCG ACC CTA TCT CTA CCG GGC G-3'). The PCR product was then cloned into Zero Blunt TOPO vector (Invitrogen) as described by the manufacturer to yield plasmid pXLW28. The nucleotide sequence of the insert was confirmed by sequence analysis. Plasmid pXLW28 was then digested with *EcoRI*, yielding a fragment of approximately 1.2 kb containing the *phoU* gene. This fragment was gel-purified and ligated into pWKS30 to yield pXLW31.

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
CFT073	Pyelonephritis isolate, <i>fim</i> ⁺ <i>pap</i> ⁺ <i>hly</i> ⁺	Mobley <i>et al.</i> (1990)
CFT073 ^{Rif}	Spontaneous rifampicin-resistant mutant of CFT073	Bahrani-Mougeot <i>et al.</i> (2002)
3A-E4	CFT073 <i>phoU</i> mutant, kanamycin resistant	Bahrani-Mougeot <i>et al.</i> (2002)
TOP10F'	F'(lacI ^q Tn10(TetR)) <i>mrcA</i> Δ(<i>mrr</i> – <i>hsdRMS</i> – <i>mrcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>φlacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara</i> – <i>leu</i>)7697 <i>galU</i> <i>galk</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
Zero Blunt TOPO	High-copy-number cloning vector, ampicillin resistant	Invitrogen
pXLW28	Contains the CFT073 <i>phoU</i> gene, including the ribosome-binding site, cloned into Zero Blunt TOPO vector, ampicillin resistant	This study
pWKS30	Low-copy-number cloning vector, ampicillin resistant	Wang & Kushner (1991)
pXLW31	pWKS30 with the 1.2 kb <i>EcoRI</i> fragment containing the CFT073 <i>phoU</i> gene, ampicillin resistant	This study

SDS-PAGE and immunoblot analysis. Whole-cell lysates were denatured by boiling for 5 min in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred to an Immobilon-P PVDF membrane using a semi-dry Multiphor II NovaBlot transfer apparatus (Amersham Pharmacia Biotech). After incubation overnight at 4 °C in blocking reagent (5 % dried skimmed milk in PBS and 0.1 % Tween 20), the membrane was probed with anti-PhoU serum (1:5000, kindly provided by J. P. M. Tommassen, Utrecht University, The Netherlands) and horseradish-peroxidase-conjugated anti-rabbit serum (Amersham Pharmacia Biotech) as the secondary antibody. The membranes were thoroughly washed and developed using the enhanced chemiluminescent detection kit (Amersham Pharmacia Biotech).

Alkaline phosphatase assay. Alkaline phosphatase activities were measured as described by Brinkman & Beckwith (1975). Briefly, strains were grown overnight in LB broth as described above. The cultures were diluted and allowed to grow in minimal MOPS medium with excess phosphate (1.32 mM) or limiting phosphate (0.10 mM) to an OD₆₀₀ of 0.5, centrifuged, washed, and resuspended in their original volume of MOPS. The cell density of each sample was confirmed by measuring the OD₆₀₀. A volume of cells (100 μl) was added to AP buffer (1 M Tris pH 8.0) to a final volume of 0.9 ml, 100 μl 0.4 % *p*-nitrophenyl phosphate (Sigma) was added, and the reaction was started by incubation at 37 °C. Reactions were stopped by adding 120 μl stop solution (1:5 mix of 0.5 M EDTA and 1 M KH₂PO₄). The activity per cell was calculated in Miller units.

Growth in human urine. Urine was collected from healthy women volunteers aged 20–40 who had no history of UTI or antibiotic use in the preceding 2 months. Each urine sample was immediately filter-sterilized and frozen at –80 °C for use within 2 weeks. For each experiment, a different pool of urine samples from three to five volunteers was used. The Institutional Review Board of the University of Maryland School of Medicine approved the study. The strain to be tested was grown overnight in LB medium with the appropriate antibiotics. The next day, bacterial cells were diluted into urine to achieve a starting OD₆₀₀ of <0.05. During incubation at 37 °C with shaking, aliquots were removed at various time points and the bacterial titres determined by plating 10-fold serial dilutions in phosphate-buffered saline in duplicate on the appropriate medium.

Mouse experimental infections. A CBA mouse model of ascending UTI was used as described by Mobley *et al.* (1990). Briefly, CBA mice were transurethrally challenged with 10⁸ c.f.u. of bacteria per

mouse. After 2 days, the mice were sacrificed and bacteria recovered from the urinary bladder, and kidneys were enumerated on plates containing appropriate antibiotics. For co-challenge infections, mice were inoculated with a mixture of 5 × 10⁷ c.f.u. of the wild-type strain CFT073 (Rif^R) and 5 × 10⁷ of the mutant (Kan^R) (total of 10⁸ c.f.u. per inoculum), which had been grown separately overnight. In these experiments, both the wild-type and mutant strain contained the vector pWKS30 or the wild-type strain contained pWKS30 while the mutant contained the complementing plasmid pXLW31. After 48 h, urine was collected; bladder and kidneys were removed, weighed and homogenized; and dilutions were plated on selective media containing rifampicin or kanamycin using a spiral plater. After overnight growth, the viable counts were determined as c.f.u. per ml urine or c.f.u. per g tissue. As the lower limit of detection was 10² c.f.u., samples yielding no colonies were scored as having this value. A competitive index was calculated for each mutant as the geometric mean of the ratios of the mutant to the wild-type strains recovered from each sample site divided by the ratios of the mutant to the wild-type strains in the inoculum.

For statistical analysis, The Mann–Whitney test was used to compare the distributions of the number of c.f.u. per ml or per g in independent infection assays. A repeated measure analysis of variance with rank order data (STATA software) was used for statistical analysis of the co-challenge experimental data as previously described (Bahrani-Mougeot *et al.*, 2002). P values of ≤0.05 were considered significant.

RESULTS

A *phoU* mutation of *E. coli* strain CFT073 results in deregulation of alkaline phosphatase

We previously used STM to identify mutants of ExPEC strain CFT073 attenuated for survival in a murine model of ascending UTI (Bahrani-Mougeot *et al.*, 2002). During this previous analysis, mutant 3A-E4 was identified as a *phoU* mutant that was significantly out-competed by the wild-type strain *in vivo*, but able to grow as well as the wild-type *in vitro*. To further characterize this mutant, we determined by sequence analysis that the transposon inserted within the *phoU* gene at the 3' end, 50 bp upstream of the stop codon. Western blot analysis was performed to determine if PhoU

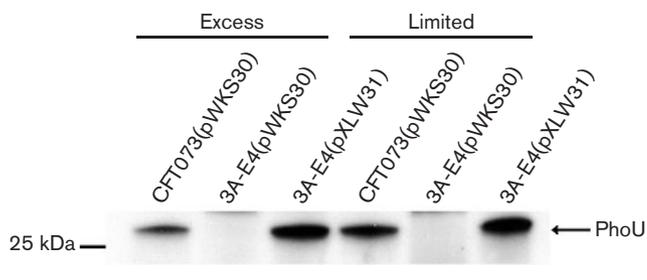


Fig. 1. Expression of PhoU by wild-type *E. coli* strain CFT073, mutant strain 3A-E4 and complemented strain 3A-E4(pXLW31). Wild-type strain CFT073(pWKS30), *phoU* mutant 3A-E4(pWKS30) and complemented mutant strain 3A-E4(pXLW31) were grown in MOPS medium containing excess (1.32 mM) or limiting (0.1 mM) phosphate. Whole-cell lysates were separated by SDS-PAGE and transferred to PVDF membranes for Western blotting with PhoU antiserum as the primary antibody and horseradish-peroxidase-conjugated anti-rabbit serum as the secondary antibody.

expression was affected by the transposon insertion. As shown in Fig. 1, the PhoU protein was present in the wild-type strain but could not be detected in the mutant strain, indicating that the transposon disrupted the expression of PhoU. Transformation of mutant 3A-E4 with plasmid pXLW31 containing the *phoU* gene, including its ribosome-binding site, restored the expression of PhoU.

Prior studies with laboratory strains of *E. coli* have shown that mutations in the *pst-phoU* operon lead to constitutively high expression of Pho regulon products, such as alkaline phosphatase. Pho regulon products are induced when bacteria are grown in limited-phosphate medium and repressed in excess-phosphate medium. Accordingly, we tested wild-type strain CFT073, *phoU* mutant 3A-E4 and complemented mutant strain 3A-E4(pXLW31) for alkaline

phosphatase activity after growth in phosphate-excess and phosphate-limiting conditions. As shown in Fig. 2, the mutant had derepressed alkaline phosphatase activity, especially under high-phosphate conditions, but this deregulation was corrected by the complementing plasmid. Restoration of alkaline phosphatase activity in the mutant to wild-type levels by pXLW31 indicates that the cloned *phoU* gene is functional. These data demonstrated *in vitro* trans complementation by the recombinant *phoU* gene, restoring PhoU expression and alkaline phosphatase activity to wild-type levels and verifying that these phenotypes were due to the *phoU* mutation.

Virulence of the *phoU* mutant strain is restored by the *phoU* recombinant plasmid

Transposon insertions into the genome can have polar effects that affect downstream genes, which may actually cause the virulence defect. In addition, unintended mutations can arise during strain manipulation. If the colonization defect of mutant 3A-E4 was indeed due to the *phoU* mutation, introducing a functional copy of *phoU* into mutant 3A-E4 should restore colonization. In initial experiments, a suspension of 10^8 c.f.u. of wild-type, mutant strain 3A-E4, or complemented mutant strain was transurethrally inoculated into the bladders of mice. After 2 days of infection, urine samples were collected from mice and used to quantify the c.f.u. per ml of urine. Mice were sacrificed and the bladders and kidneys were removed and used to determine the c.f.u. per g tissue for each strain. When administered alone, the *phoU* mutant strain and the complemented *phoU* mutant strain were able to colonize the mouse urinary tract at levels comparable to and not significantly different than those of the wild-type strain CFT073 (Fig. 3). Thus, PhoU does not appear to be required for colonization of the murine urinary tract in experiments involving separate inoculation. However, this result does

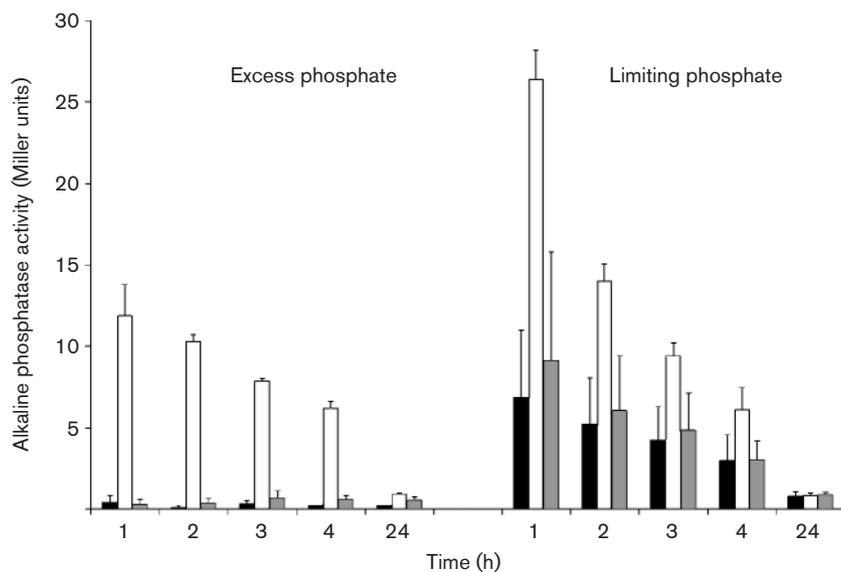


Fig. 2. A *phoU* mutant of *E. coli* strain CFT073 has derepressed alkaline phosphatase activity. Bacteria were grown in MOPS medium containing excess or limiting phosphate to mid-exponential phase, centrifuged, washed, and resuspended in their original volume of MOPS. An aliquot of cells was added to AP buffer followed by the addition of 0.4% *p*-nitrophenyl phosphate. The reaction was incubated at 37 °C and stopped at the indicated time points. The alkaline phosphatase activity per cell was calculated in Miller units. Wild-type *E. coli* strain CFT073 is shown in black columns, *phoU* mutant strain 3A-E4 in white columns, and complemented mutant strain 3A-E4(pXLW31) in grey columns. Error bars indicate SEM from two experiments with triplicate values.

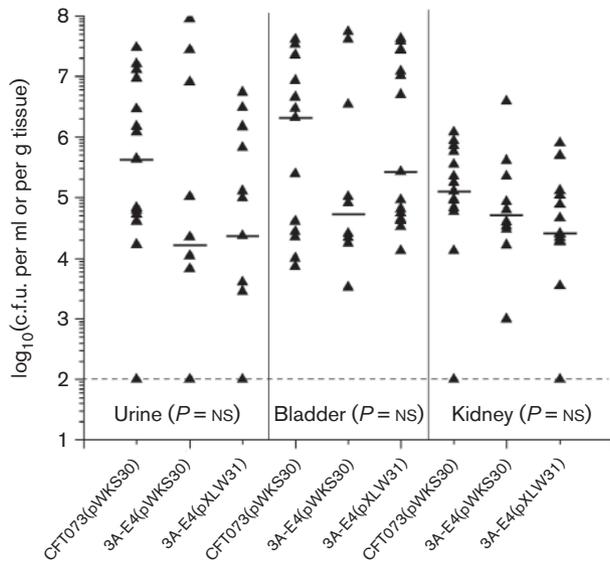


Fig. 3. PhoU is not required for colonization of the murine urinary tract. Groups of 10 mice were transurethraly challenged with the wild-type strain CFT073(pWKS30), *phoU* mutant 3A-E4(pWKS30) or complemented mutant strain 3A-E4(pXLW31). After 2 days, bacteria were recovered from urine, bladder and kidneys, and c.f.u. were enumerated on selective medium. Each symbol represents the c.f.u. per ml urine or c.f.u. per g tissue from an individual mouse. The lower limit of detection (10^2) in this assay is indicated by the dashed line. Horizontal bars represent the median of the colony counts. Differences between the strains were not statistically significant.

not exclude a subtle role for PhoU that cannot be detected under these conditions.

We next performed competition colonization experiments in which the wild-type CFT073 and the mutant or complemented mutant strain were administered together in a 1:1 ratio to individual mice. Competition colonization experiments eliminate the variation between animals that is inherent in the single-infection experiments and thus are a more sensitive indicator of the ability of a mutant to survive *in vivo* compared to the wild-type strain. After 2 days of infection, bacteria were recovered from urine, bladder and kidneys, and c.f.u. were enumerated on selective medium. As shown in Fig. 4(a), the *phoU* mutant strain was recovered at significantly lower levels than wild-type CFT073 in the kidneys and urine when both strains contained the plasmid vector control pWKS30. Levels of colonization of the bladder by the *phoU* mutant tended to be lower than those of colonization by wild-type strain CFT073; however, the difference was not significant. Overall, the *phoU* mutant strain was out-competed by the wild-type strain in the bladder, kidneys and urine when considered together ($P < 0.001$). When the *phoU* mutant strain was complemented with pXLW31, the ability to compete successfully with the wild-type was restored (Fig. 4b). The complemented strain was

recovered from the urine, kidneys and bladder in numbers comparable to those of the wild-type strain. These results indicate that the ability to produce PhoU is critical to allow ExPEC strain CFT073 to compete for colonization of the murine urinary tract.

Effect of PhoU on growth in human urine

It has been suggested that a mutation in *phoU* can cause a severe growth defect due to the accumulation of phosphate, and this growth defect can be alleviated by a compensatory mutation in the *pstSCAB* genes or in the *phoBR* genes (Steed & Wanner, 1993). However, sequencing of the *pstSCAB* and the *phoBR* genes of the *phoU* mutant revealed no mutation within these genes compared to the published sequence from the wild-type strain. To verify that inactivation of *phoU* did not affect growth of the mutant, we compared the growth of the wild-type parent CFT073 with the *phoU* mutant in LB broth. We detected no difference between the wild-type and mutant strains when grown separately in LB broth (not shown). Similarly, when the two strains, each containing a control plasmid, were inoculated together in LB, they grew at similar rates (Fig. 5a). Except for the 6 h time point, when the wild-type strain outcompeted the mutant, there were no significant differences in colony counts between the strains at any time point. When the wild-type containing the control plasmid and the mutant strain complemented with a plasmid containing the *phoU* gene were grown together in LB, there were no significant differences at any time point (Fig. 5b). This result suggests that the *phoU* mutation had only a subtle effect on the growth of CFT073 in laboratory media. Similar results were obtained when the wild-type and mutant strains were grown separately in human urine *in vitro* (data not shown). However, in competition growth experiments in human urine, the wild-type out-competed the *phoU* mutant during the entire growth period when both strains contained the control plasmid (Fig. 5c). The differences between the wild-type and mutant strains reached statistical significance at 4 and 6 h. This difference was reduced, but not eliminated, by complementation (Fig. 5d); none of these differences were statistically significant. These results suggest that mutation of *phoU* results in a competitive disadvantage for growth that is most apparent in human urine and the murine urinary tract.

DISCUSSION

The human urinary tract represents a unique host environment in which ExPEC strains can survive and grow. Urine and cellular components in the urinary tract are likely to serve as signals for the increased expression of a variety of genes that aid in the survival of these strains (Snyder *et al.*, 2004). In addition, environmental signals such as temperature, nutrient availability, pH, osmolarity and starvation for phosphate have all been shown to influence bacterial virulence gene expression. Previously, we identified a *phoU* mutant of ExPEC strain CFT073 and demonstrated that this

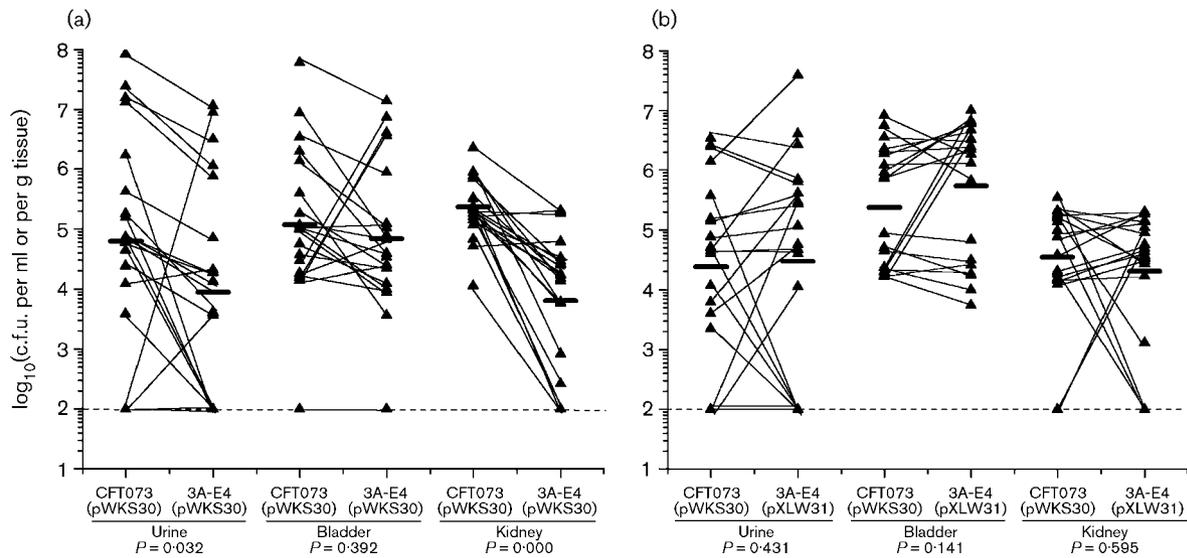


Fig. 4. PhoU is required for competition with the wild-type strain for efficient colonization of the murine urinary tract. Competition colonization experiments were performed with wild-type strain CFT073(pWKS30) and *phoU* mutant 3A-E4(pWKS30) (a) or CFT073(pWKS30) and complemented *phoU* mutant 3A-E4(pXLW31) (b). Groups of 10 mice were transurethrally challenged with a 1:1 mixture of the wild-type strain and the mutant strain. After 2 days, bacteria were recovered from urine, bladder and kidneys, and c.f.u. were enumerated on selective medium. Each symbol represents the c.f.u. per ml urine or c.f.u. per g tissue from an individual mouse. Lines connect values from the same mouse. The lower limit of detection (10^2) in this assay is indicated by the dashed line. Horizontal bars represent the median of the colony counts of two independent experiments. *P* values are indicated.

mutant was attenuated for survival in the murine urinary tract. This finding suggested that the PhoU protein might serve as a urovirulence factor by indirectly affecting the expression of virulence-related genes, leading to attenuation. In this report, we demonstrate that PhoU enhances the ability of prototype ExPEC strain CFT073 to survive and to colonize the murine urinary tract. Thus PhoU joins a shortlist of confirmed urovirulence factors.

The role of PhoU in CFT073 colonization of the urinary tract was assessed by comparing the *phoU* mutant with wild-type strain CFT073 in the murine model of ascending UTI. In single-infection assays, quantitative cultures of urine, bladder and kidney revealed no difference between the wild-type strain CFT073 and the *phoU* mutant strain in the ability to colonize the mouse urinary tract. However, in competitive colonization experiments, the *phoU* mutant strain was significantly out-competed by the wild-type strain in the kidneys and urine and recovered in lower amount in the bladder. Importantly, reintroduction of the *phoU* gene into the mutant abolished this difference, proving that the PhoU itself is responsible for this competitive advantage.

Other members of the *pst* operon have been suggested to be important in the virulence of various pathogens. Recently, Burall *et al.* (2004) identified attenuated mutants with insertions in the *pstS* and *pstC* genes by STM of uropathogenic *Proteus mirabilis*. A mutation in *pstC* rendered a strain of *E. coli* that causes bacteraemia in pigs both avirulent and

serum sensitive (Daigle *et al.*, 1995). The *pstS* gene of *Salmonella enterica* serovar Typhimurium was identified as a macrophage-inducible gene and a mutation in *pstS* was shown to reduce the expression of *hilA* and invasion genes (Lucas *et al.*, 2000). Polissi *et al.* (1998) identified a *pstB* mutant in a screen of *Streptococcus pneumoniae* mutants incapable of surviving in a bacteraemia model. However, to our knowledge, this is the first time that the importance of the *pst-phoU* operon in virulence has been confirmed using genetic complementation. As in our study, the specific mechanism by which PhoU and other products of the *pst-phoU* operon contribute to pathogenesis has not been elucidated in these other pathogens.

Although the role of PhoU within the Pho regulon is not fully understood, it seems to act as a negative regulator. Prior work and our results confirm that *phoU* mutation leads to increased expression of genes normally repressed under conditions, including urine, where P_i is abundant. This inappropriate expression of unnecessary proteins could place an extra burden on a *phoU* mutant, a burden that does not result in a measurable difference in growth rate, but one that is manifest under the more stringent conditions of *in vitro* or *in vivo* competition for growth with a strain that has an intact Pho regulon. It is noteworthy that this defect was more obvious during growth in human urine than in laboratory medium. In an attempt to pinpoint specific metabolic defects that could result in this competitive disadvantage, we compared the *phoU* mutant with the

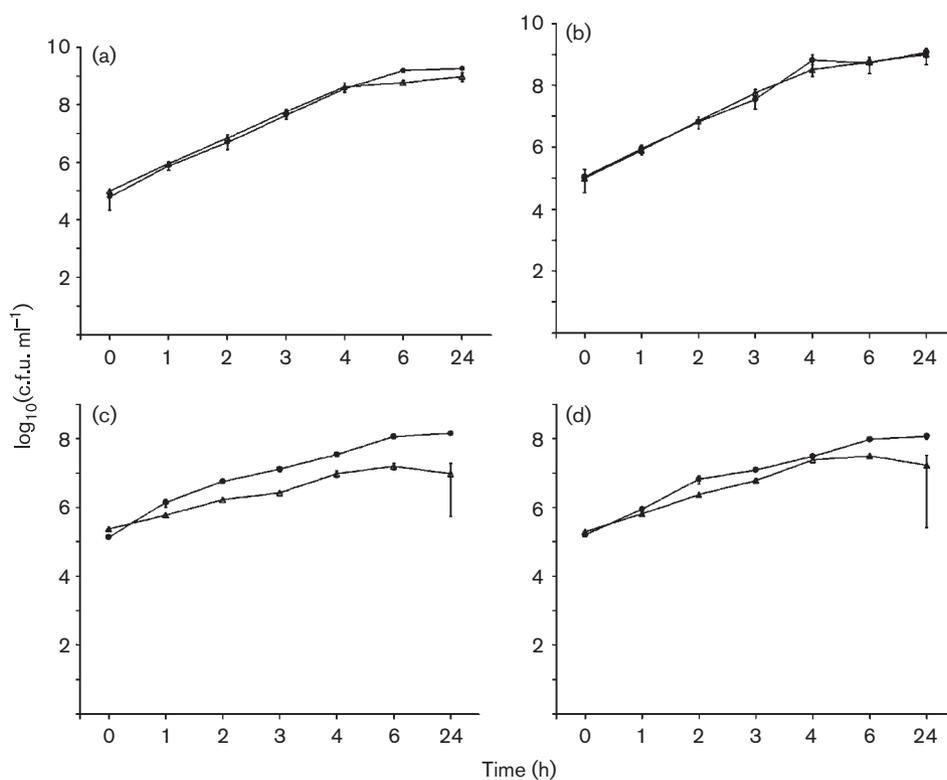


Fig. 5. Competitive growth and survival of the *phoU* mutant in LB broth and human urine. Human urine was collected as described in Methods. Bacterial cells were diluted into LB broth (a, b) or human urine (c, d) and incubated at 37 °C. Viable counts (c.f.u. ml⁻¹) were determined at different time points by plating on selective medium. ●, Wild-type *E. coli* strain CFT073; △, *phoU* mutant strain 3A-E4. In (a) and (c), both strains contained the control plasmid pWKS30; in (b) and (d), the wild-type contained pWKS30 and the mutant contained the complementing plasmid pXLW31. Data are the means \pm SEM of three independent experiments (some of the error bars are smaller than the symbols). Note that the scale on the x-axis is not linear.

wild-type strain in both phenotypic (Bochner *et al.*, 2001) and transcript (Snyder *et al.*, 2004) microarrays. However, we could not confirm any specific defect resulting from the *phoU* mutation that could account for this competitive disadvantage (data not shown). Thus, the precise mechanism by which dysregulation of the Pho regulon leads to a defect in colonization of the murine urinary tract remains to be uncovered.

ACKNOWLEDGEMENTS

We thank Richard Hebel for providing statistical analysis and Jan Tommassen for supplying the PhoU antiserum. This work was supported by NIH Program Project grant no. 2P01 DK49720 and, in part, by the Department of Veterans Affairs, Baltimore, MD. E. L. B. was supported by the United Negro College Fund/Merck Postdoctoral Fellowship.

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