RESEARCH ARTICLE

The high-affinity phosphate transporter Pst is a virulence factor for *Proteus mirabilis* during complicated urinary tract infection

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Abstract

Proteus mirabilis is a ubiquitous bacterium associated with complicated urinary tract infection (UTI). Mutagenesis studies of the wild-type strain HI4320 in the CBA mouse model of ascending UTIs have identified attenuated mutants with transposon insertions in genes encoding the high-affinity phosphate transporter Pst (*pstS*, *pstA*). The transcription of the *pst* operon (*pstSCAB-phoU*) and other members of the phosphate regulon of *Escherichia coli*, including alkaline phosphatase (AP), are regulated by the two-component regulatory system PhoBR and are repressed until times of phosphate starvation. This normal suppression was relieved in *pstS*::Tn5 and *pstA*::Tn5 mutants, which constitutively produced AP regardless of growth conditions. No significant growth defects were observed *in vitro* for the *pst* mutants during the independent culture or coculture studies in rich broth, phosphate-limiting minimal salts medium, or human urine. Mutants complemented with the complete *pst* operon repressed AP synthesis *in vitro* and colonized the mouse bladder in numbers comparable to the wild-type strain HI4320. Therefore, the Pst transport system imparts a significant *in vivo* advantage to wild-type *P. mirabilis* that is not required for *in vitro* growth. Thus, the Pst transporter has satisfied molecular Koch's postulates as a virulence factor in the pathogenesis of urinary tract infection caused by *P. mirabilis*.

Introduction

Proteus mirabilis, a Gram-negative enterobacterial species, causes a variety of diseases including pneumonia, septicemia, and wound infections, but is most commonly the cause of complicated urinary tract infections (UTIs) (Mobley, 1996). Individuals at higher risk for complicated UTI caused by this pathogen include those with long-term indwelling catheterization as well as those with structural and functional abnormalities within the urinary tract (Mobley, 1996). Clinical syndromes associated with *P. mirabilis* include cystitis and pyelonephritis with possible complications from stone formation and bacteremia (Mobley, 1996). These organisms are capable of surviving in the urinary tract to cause persistent UTIs because of their ability to produce several virulence factors [urease, flagella, fimbriae, hemolysin, IgA protease (Musher *et al.*, 1975; Peerbooms *et al.*, 1984;

Mobley *et al.*, 1994; Belas, 1996; Walker *et al.*, 1999)] and by forming crystalline biofilms on indwelling catheters (Stickler *et al.*, 1993). Currently, the complete picture of the pathogenesis of *P. mirabilis* during UTIs is not fully understood.

To better understand how these uropathogens infect the urinary tract, Burall *et al.* (2004) used signature-tagged mutagenesis to identify previously unknown virulence factors of the urinary clinical isolate *P. mirabilis* HI4320 that were required for colonization of the CBA mouse model of UTI. Of the 18 highly attenuated mutants (a $\geq 10^4$-fold difference as compared with the wild type) that were assessed by cochallenge, two of these mutants possessed transposon insertions within genes of the the *pst* (phosphate-specific transport) operon. The mutations occurred in genes that were identified as homologues of the *Yersinia pestis* high-affinity phosphate transporter periplasmic binding protein, PstS, and the high-affinity phosphate permease,

PstA (previously misidentified as PstC) (Burall *et al.*, 2004). No common *in vitro* phenotype had been determined for these signature-tagged mutants and, to date, the *pst* operon has yet to be characterized for *P. mirabilis*.

The Pst system of *Escherichia coli*, a multiprotein, high-affinity inorganic phosphate (P_i) transporter, is induced at limiting extracellular P_i concentrations ($< 1\,mM$, K_m^{app} of $0.2\,\mu M$) (Willsky & Malamy, 1980; Harris *et al.*, 2001) and compensates for the less effective transport of P_i through the low-affinity Pit protein (K_m^{app} of $11.9–38\,\mu M$) (Willsky & Malamy, 1980; van Veen *et al.*, 1994). This transporter, a member of the ATP-binding cassette (ABC) family of transporters (Ames, 1986), consists of five polypeptides. Four of the gene products are associated with phosphate transport: PstS, the periplasmic phosphate binding protein; PstC and PstA, the inner membrane permease; and PstB, the ATPase that provides energy for transport. The last polypeptide, PhoU, works with the other Pst gene products to act as a negative regulator of the phosphate (*pho*) regulon (Torriani-Gorini, 1994).

The expression of the *E. coli pho* regulon, a collection of at least 38 different genes that are induced during times of phosphate limitation (Wanner, 1993), is regulated by the two-component regulatory system PhoBR upon the detection of low environmental phosphate levels (VanBogelen *et al.*, 1996). Most of the genes that are known to be part of the *pho* regulon include those involved in transport of P_i and phosphate-containing complexes through the outer membrane (*phoE*), the degradation of those complexes in the periplasm by enzymes [alkaline phosphatase (AP), *phoA*], and the transport and assimilation of different organic (*phnCDEFGHIJKLMNOP*, *phnRSTUVWX*, *ugpBAECQ*) and inorganic phosphate (*pst* operon) sources (Wanner & Boline, 1990; VanBogelen *et al.*, 1996).

Previous studies have demonstrated a role of the Pst transport system in pathogenesis, including roles in the regulation of biofilm formation (Monds *et al.*, 2001), invasion (Sinai & Bavoil, 1993; Lucas *et al.*, 2000; Mathew *et al.*, 2001), antibiotic resistance (Soualhine *et al.*, 2005), and colonization (Daigle *et al.*, 1995; Orihuela *et al.*, 2001; Lamarche *et al.*, 2005; Peirs *et al.*, 2005; Runyen-Janecky *et al.*, 2005; Buckles *et al.*, 2006). Because mutants in this transport system were found to be attenuated upon cochallenge in the mouse model of ascending UTI and previous studies have alluded to its role in pathogenesis, the role of *pst* operon in the virulence of *P. mirabilis* during UTI has been analysed. To determine the role of this system in the pathogenesis of UTIs caused by *P. mirabilis*, this study focused on sequencing and analyzing the complete *pst* operon from *P. mirabilis* HI4320, determining phenotypes for mutants in this transport system, and complementing these mutants to verify that the *pst* operon was responsible for the phenotypes observed *in vitro* and attenuation *in vivo*.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *P. mirabilis* HI4320 was isolated from the urine of a long-term catheterized elderly woman with significant bacteriuria ($\geq 10^5\,CFU\,mL^{-1}$) (Warren *et al.*, 1982; Mobley & Warren, 1987). Wild-type HI4320 possessing pBAC001 was utilized for constitutive expression of GFP as described previously (Jansen *et al.*, 2003). The *pst* mutants used in this study, G1-43 (*pstA*::Tn5) and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
P. mirabilis strains		
HI4320 WT	Wild-type *P. mirabilis* isolate, Tet[r]	Mobley Warren (1987)
G1-43 (PMH*pstA*)	*pstA* mutant of HI4320 (*pstA*::Tn5 Kan[r])	Burall *et al.* (2004)
H4-34 (PMH*pstS*)	*pstS* mutant of HI4320 (*pstS*::Tn5 Kan[r])	Burall *et al.* (2004)
Plasmids		
pBAC001	Used to distinguish HI4320 from *pst* mutants for confocal microscopy, 3840 bp, pBAC[TM] vector, ColE1 ori, constitutively expressing GFP by *gfpmut2* gene, Amp[r]	Jansen *et al.* (2003)
pCR2.1-TOPO	Cloning PCR fragments, 3931 bp, f1 & pUC ori, *lacZα*, Amp[r]Kan[r], screening by blue-white colonies	Invitrogen
pCRXL-TOPO	Cloning large PCR fragments (3–10 kb), 3519 bp, pUC ori, *lacZα*, Zeocin[r]Kan[r], *cedB* lethal gene, screening by blue-white colonies	Invitrogen
pKHKS403	Moderate-copy-number cloning vector used for complementation, 3159 kb, pBluescript with p15A ori from pACYC184, Amp[r]	Provided by H. Mobley
pSMJ001	pKHKS403 with *pstS*	This study
pSMJ002	pKHKS403 with *pstA*	This study
pSMJ003	pKHKS403 with *pstS-phoU* (*pst* operon)	This study

H4-34 (*pstS*::Tn5), were generated in a previous study by signature-tagged mutagenesis (Burall *et al.*, 2004). All plasmids were initially constructed in either *E. coli* DH5α (Bethesda Research Laboratories) or *E. coli* TOP10 (Invitrogen).

Luria broth (containing, per liter, 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) and nonswarming agar (containing, per liter, 10 g of tryptone, 5 g of yeast extract, 5 mL of glycerol, 0.4 g of NaCl, and 20 g of agar) were routinely used with the appropriate antibiotics (15 μg mL^{-1} tetracycline, 50 μg mL^{-1} kanamycin, and/or 100 μg mL^{-1} ampicillin) to culture bacteria. The phosphate-limiting minimal salts medium (1 μM, pH 7.2, containing, per liter, 0.47 g of sodium citrate, 1.0 g of $(NH_4)_2SO_4$, 72 μL of 0.01 M K_2HPO_4, 28 μL of 0.01 M KH_2PO_4, 1.0 mL of 1 M $MgSO_4$, 10 mL of 20% glycerol, and 1.0 mL of 1.0% nicotinic acid) utilized in this study is a variation of the phosphate-containing minimal salts medium described previously (Belas *et al.*, 1991). For use of human urine as a growth medium, urine was collected from multiple anonymous healthy donors, pooled, filter sterilized and stored in the dark at 4.0 °C until use. Normal adult human urine is a high phosphate medium as it possesses *c.* 0.9–1.3 g of phosphate per 24 h (www.webmd.com). With normal urine output being *c.* 800–2000 mL day^{-1} (www.nlm.nih.gov/), there is *c.* 0.45–1.625 M of urinary phosphate per day or 0.045–0.1625 g phosphate mL^{-1} urine day^{-1}.

Sequence determination of *pst* operon in *P. mirabilis* HI4320

The initial verification of the two *pst* mutations has been described previously by the authors' laboratory (Burall *et al.*, 2004). In this study, before the sequencing of the *P. mirabilis* HI4320 genome by the Sanger Institute, the sequence of the *pst* operon was determined for *P. mirabilis* HI4320 using arbitrary and standard PCR with *Taq* DNA polymerase (Roche). PCR fragments were electrophoresed on a 1% agarose gel, excised, and purified using the Qiaquick gel extraction protocol (Qiagen). DNA sequencing was performed by the Biopolymer Laboratory at the University of Maryland, School of Medicine, using the dideoxy chain termination method with double-stranded DNA as the template. DNA sequencing reactions were processed by a 16-capillary automated DNA sequencer (model 3100; Applied Biosystems). The BLASTN and B12SEQ ALIGNMENT program (NCBI, http://www.ncbi.nlm.nih.gov) were used to analyze and assemble the sequences obtained from the sequencing core. Amplification and sequencing primers used for this study are listed in Table 2.

Nucleotide and protein sequence analysis

The DNA sequence upstream of the translational start site of *pstS* was scanned for core promoter elements (the Pribnow box or − 10 element and − 35 element), a ribosomal binding site, as well as known consensus sequences, such as the Pho box, which has been shown to be important for regulation of the *E. coli pst* operon (Shine & Dalgarno, 1974; Pribnow, 1975; Makino *et al.*, 1986; Kimura *et al.*, 1989). Computer analysis of the genes and gene products of the *pst* operon was accomplished using several computer analysis programs. The molecular weight and pI predictions were carried out using the Compute pI/MW Tool located at ExPASy Molecular Biology Server (http://us.expasy.org/). The cellular location of the Pst protein homologues was predicted using PSORT-B [http://www.psort.org/, (Gardy *et al.*, 2003)]. Determination of the potential function of the Pst homologues was accomplished by predicting putative protein domains [the Simple Modular Architecture

Table 2. Primers used in this study

Primers	Sequence	Description
Arb-1	GGCCACGCGTCGACTAGTCA(N)10	Arbitrary PCR
Arb-2	GGCCACGCGTCGACTAGTCA	Arbitrary PCR
M13 forward	GTAAAACGACGGCCAG	PCR fragment insert from pCR2.1
M13 reverse	CAGGAAACAGCTATGAC	PCR fragment insert from pCR2.1
Mob2138 (G1-43 out)	GCTAGTCGAACTAGTGCGCT	1015 bp fragment of intragenic region between the *pstS* and *pstC* genes
Mob2141 (H4-34 out)	GCATAGGCTCTTCTGGTGGTG	1015 bp fragment of intragenic region between the *pstS* and *pstC* genes
Mob2170 (*pstS* in)	GCTATCTGCCCATTTTGCAT	860 bp fragment of the 5′ end of *pstS* gene and upstream sequence
Mob2171 (*pstS* out)	GCAATGATCTGTTTTACACCA	Larger fragment used as template for second reaction in arbitrary PCR to determine the promoter region of *pstS* gene
Mob2372 (*pstS* 5′)	CGTACGAATTCCGAAGTTGATAGATG	Isolate *pstS* intact gene for complementation
Mob2373 (*pstS* 3′)	GCCTCAATTCTTTATTACCCCCTGTGTTGC	Isolate *pstS* intact gene for complementation
Mob2395 (*pstA* 5′)	GGAATTAACATCCTCTTCTTC	Isolate *pstA* intact gene for complementation
Mob2396 (*pstA* 3′)	TGAATTACTTTCTGCGTTAGC	Isolate *pstA* intact gene for complementation
Mob2397 (*pst* operon 5′)	CGTACCGAATTCGAA	Isolate intact *pst* operon for complementation
Mob2398 (*pst* operon 3′)	GCCTATCACGAATTCAAGTTG	Isolate intact *pst* operon for complementation

Research Tool (SMART) server at EMBL (http://smart.embl-heidelberg.de/)] and putative transmembrane domains [SMART server, the DAS transmembrane prediction server (http://www.sbc.su.se/~miklos/DAS/maindas.html (Cserzo *et al.*, 1997))].

Rapid verification of previously sequenced regions and identification of genes known to be associated with phosphate assimilation and transport (*phnABCDEFGHIJKLM-NOPRSTUVWX, phoA, phoBR, phoE, phoH/psiH, pitA, pitB, ppk, ppx/gppA, pstSCAB-phoU, ugpBAECQ*) was determined by analyzing the genome sequence of *P. mirabilis* HI4320, which was recently completed by the Sanger Centre (http://www.sanger.ac.uk/Projects/P_mirabilis/). Potential homologues were identified using the BLAST server provided by the Sanger Centre (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/p_mirabilis). Sequences homologous to *P. mirabilis* were subsequently examined by an ORF finder program (NCBI, http://www.ncbi.nih.gov/gorf/gorf.html). The translated protein products of the predicted ORFs were then analyzed for their homology to known protein sequences for other prokaryotes (*Y. pestis* KIM, uropathogen *E. coli* CFT073, *Salmonella typhimurium* LT2, and *Photorhabdus luminescens* sp. *laumondii* TT01) using BLAST (NCBI). The predicted *P. mirabilis* HI4320 homologues have not yet been assigned accession numbers.

Growth studies

Independent growth in Luria broth, phosphate-limiting minimal salts medium and human pooled urine was evaluated over time for wild-type strain HI4320 and the *pst* mutants. Overnight cultures of each strain were inoculated into fresh Luria broth containing no antibiotic and incubated at 37 °C until an $OD_{600\,nm}$ of 0.1 was reached. This standardized culture was used to inoculate the test medium; $OD_{600\,nm}$ was recorded every hour for the first 6 hours and after 24 and 48 h.

Cocultures were performed as described previously (Burall *et al.*, 2004). Briefly, overnight cultures were inoculated into fresh Luria broth and incubated at 37 °C with shaking until the $OD_{600\,nm}$ reached 1.0. A 1 : 1 mix consisting of standardized cultures of wild-type and either *pst* mutant was used to initiate the competition. Cultures were repassaged into fresh medium without antibiotics twice (Luria broth) or once (phosphate-limiting medium) a day for up to 4 days. Viable counts (in $CFU\,mL^{-1}$) were determined for the input time point and subsequent time points by plating dilutions onto plain Luria agar plates with and without kanamycin. Wild type counts were obtained by subtracting the $CFU\,mL^{-1}$ of the Luria agar plates with kanamycin from the $CFU\,mL^{-1}$ of the plain Luria agar plates. The limit of detection of this assay is $10^2\,CFU$. For statistical analysis, plates with no counts were adjusted to this value. A growth

defect was ruled out if the *pst* mutants competed successfully with the parent strain.

To better mimic the nutritional environment of the urinary tract, additional *in vitro* competition studies were conducted using pooled human urine as a growth medium. Previous *in vitro* competition studies using urine as a growth medium have produced contrasting results, likely due to the association of *P. mirabilis* HI4320 with rapidly forming crystals, which in turn hampers the isolation of individual colonies and the ability to obtain accurate viable cell counts. Therefore, to better differentiate between the bacterial strains and crystalline structures, enumeration of the wild-type and mutant bacteria in coculture was accomplished through differential staining and imaging via laser scanning confocal microscopy. Overnight cultures inoculated into fresh Luria broth were standardized to an $OD_{600\,nm}$ of 0.1. A 1 : 1 mixture of wild-type containing pBAC001 and either *pst* mutant was used to initiate the competition. The cultures were repassaged into fresh urine without antibiotics once a day for up to 4 days. After 0, 1, 2, 3, and 4 days of coculture, samples were pelleted and resuspended in 0.85% NaCl. Then, bacteria were stained with $1.0\,\mu L^{-1}$ Cell Tracker Orange CMRA (Molecular Probes) for detection of live cells and $2.0\,\mu L^{-1}$ ToPro 3 (Molecular Probes) for detection of dead cells. The green fluorescent protein (GFP)-producing plasmid, pBAC001, was used to differentiate wild-type from the *pst* mutants and has been shown to be stable during growth in the urinary tract and *in vitro* (Jansen *et al.*, 2003). After staining, samples were washed with 0.85% NaCl and resuspended in a final volume of $50\,\mu L$. Of this final volume, $10\,\mu L$ was placed onto a glass slide (FisherBrand Premium slide) with a coverslip. Samples were viewed using an LSM510 Meta laser scanning confocal microscope (Carl Zeiss Inc.) equipped with a Plan-Apochromat $\times 100/1.4$ oil DIC lens. Bacteria were enumerated using the LSM 510 META IMAGE ACQUISITION software (Zeiss). Wild type cells were denoted as green and *pst* mutants as red. The percentage of each strain present within the population at each time point was calculated. To ensure plasmid stability during the *in vitro* competition, the GFP-producing wild-type was passaged independently in pooled human urine over a 4-day period and the percentage of cells that were no longer producing GFP was determined for each day of the passage. These percentages were taken into consideration in the final calculations reported.

AP assay

AP activity was measured using a variation of the method of Brickman & Beckwith (1975). Briefly, overnight cultures of each strain were inoculated into fresh medium (Luria broth, phosphate-limiting minimal salts medium, or human pooled urine) without antibiotic and incubated for *c.* 2 h at

37 °C with shaking. Cultures were standardized to an $OD_{600\,nm}$ of 0.1, harvested by centrifugation, and then pelleted and resuspended in 1.0 M Tris, pH 8.0. The $OD_{600\,nm}$ of the resuspended sample was recorded. The AP reaction started with the addition of 0.4% *p*-nitrophenyl-phosphate (*p*-NPP) and was stopped with the addition of 1.0 M K_2HPO_4. Once the $OD_{405\,nm}$ and $OD_{550\,nm}$ measurements were recorded, the phosphatase units (U) were calculated utilizing a formula developed by J. H. Miller for determining units of β-galactosidase [$PU = 1000 \times (OD_{405\,nm} - 1.75\,(OD_{550\,nm})/($Time of reaction (min) $\times$ volume of culture (mL) $\times OD_{600\,nm})$] (Miller, 1972). The limit of detection of the assay was 0 PU. Therefore, for statistical analysis, any negative measurement was adjusted to this value.

Complementation of the *pst* mutants

The *pstS* and *pstA* genes were amplified by PCR using wild-type *P. mirabilis* HI4320 chromosomal DNA as template (primers listed in Table 2). The entire *pst-phoU* operon was amplified using Expand Long PCR (Roche) as directed by the manufacturer's instructions. All PCR fragments were initially inserted into either pCR®2.1-TOPO (*pstS* and *pstA* genes) or pCR®XL-TOPO (*pst-phoU* operon) and transformed into either electrocompetent *E. coli* DH5α or chemically competent *E. coli* TOP10. The resulting cloned fragments were confirmed by restriction digestion and sequencing. Fragments were subsequently ligated into the moderate copy plasmid, pKHKS403. The pKHKS403 vector containing either the individual *pst* genes, the *pst* operon, or the vector alone was cloned into *E. coli* DH5α. The plasmids were isolated and electroporated into *P. mirabilis* HI4320 wild-type and respective *pst* mutants. All clones containing the various plasmids were selected for by ampicillin resistance. The clones were prescreened for the lack of detectable AP activity and the plasmids from these clones were isolated and subsequently digested by EcoRI to confirm insertion of the PCR fragment. To verify *in vitro* complementation of the *pst* mutants, a comparison of the AP activity of wild-type, the *pst* mutants, and the mutants containing the complementing or vector control plasmids was conducted as described previously with cultures grown for 17 h with shaking at 37 °C in Luria broth. *In vivo* complementation was examined by determining whether the addition of the complementing plasmid pSMJ003 restored the amount of *pstA*::Tn5 mutant colonization to wild type levels during cochallenge of CBA/J mice.

CBA mouse model of ascending UTIs

A modification (Johnson *et al.*, 1987) of the mouse model of ascending UTI originally developed by Hagberg *et al.* (1983) was used. For the *in vivo* complementation study, wild type

transformed with the empty pKHKS403 vector and the *pstA*::Tn5 mutant transformed with the complementing plasmid pSMJ003 were grown in Luria broth with ampicillin (50 µg mL⁻¹) at 37 °C, shaking for 16 h. Cultures were then pelleted, resuspended in phosphate-buffered saline (pH 7.4, Sigma) and standardized to the same $OD_{600\,nm}$. Five female CBA/J mice (20–22 g, 6–8 weeks old; Harlan Sprague Dawley, Indianapolis, IN) were anesthetized with a 20 mg mL⁻¹ ketamine 3 mg mL⁻¹ xylazine solution and in-oculated transurethrally with 50 µL of a 1 : 1 bacterial suspension of the HI4320 wild-type (pKHKS403) and *pstA*::Tn5 (pSMJ003). To determine the input CFU mL⁻¹ of each strain, dilutions of this inoculum were spiral plated using an Autoplate 4000 (Spiral Biotech) and yielded *c*. 2×10^7 CFU mouse⁻¹. Mice were provided with drinking water containing ampicillin (250 µg mL⁻¹) 3 days before and throughout infection to maintain selective pressure on bacterial plasmids. After 48 h postinfection (hpi), urine was collected and the mice were sacrificed by overdose with isoflurane. The bladder was aseptically removed, weighed, and homogenized. Undiluted and diluted (1 : 10) urine and homogenized tissue were spiral plated onto Luria agar plates containing ampicillin (50 µg mL⁻¹) with or without kana-mycin (25 µg mL⁻¹). Wild type counts were obtained by subtracting the CFU mL⁻¹ of Luria agar plates containing ampicillin and kanamycin from the CFU mL⁻¹ of the Luria agar plates containing ampicillin alone. All spiral plates were read using a Q-Count machine and software (Spiral Bio-tech) to determine bacterial concentrations. The limit of detection of this assay is 10^2 CFU mL⁻¹ of urine or g⁻¹ tissue. Therefore, for statistical analysis, plates with no counts were adjusted to this value.

Statistical analysis

A Student's *t* test was used to determine significant differences in growth throughout all of the *in vitro* growth studies. Because the data for the *in vivo* cochallenge experiments were generally nonparametric, significant differences between the numbers of wild-type and mutant CFU recovered throughout infection were determined using a repeated-measure ANOVA with rank order data (a ranked-sum test, STATA software; Stata Co.). For all studies, a *P* value < 0.05 was considered significant.

Results

Signature-tagged *pst* mutants of *P. mirabilis* HI4320 are attenuated in the mouse model of UTI

Signature-tagged mutagenesis studies of *P. mirabilis* HI4320, conducted previously in the laboratory (Burall *et al.*, 2004), demonstrated that mutation of *pstS* or *pstA* resulted in outcompetion of the mutants in the urine, bladder, and

kidneys by $\geq 10^3$-fold during cochallenge of mice with the parent strain (Fig. 1a and b). Because that study indicated a potential role of the *pst* operon in virulence, further characterization of the Pst system in *P. mirabilis* HI4320 was warranted to determine whether Pst-mediated phosphate transport is required for *Proteus* pathogenesis.

Proteus mirabilis HI4320 has an intact *pst* operon

The *pst* operon was sequenced as described in 'Materials and methods' and was verified using the recently assembled nucleotide sequence of *P. mirabilis* HI4320 (M. Pearson, M. Sebaihia, J. Parkhill, H. Mobley, in preparation). We determined that *P. mirabilis* HI4320 possesses an intact *pst* operon organized similarly to that observed in other bacterial species (*pstSCAB-phoU*) (Fig. 1c). Analysis of the nucleo-

tide sequence upstream of the first gene of the operon, *pstS*, predicted that this region contains a ribosomal-binding site AGGAGG, a – 10 element (TATTGT), and two Pho boxes (TTGTCAT-AAAA-CTGTCAC-AATT-AAGACAT-TTAA-TT GTCAT). The predicted cellular location, potential function, isoelectric point, and molecular weight of each Pst protein of HI4320 are listed in Table 3.

Gene homologues associated with phosphate assimilation and transport in *P. mirabilis* HI4320

The Pst system is a member of the *pho* regulon, a group of genes involved in the transport and assimilation of P_i. To determine whether *P. mirabilis* HI4320 possesses a *pho* regulon that is similar to those observed for other organisms (*E. coli* CFT073, *Y. pestis* KIM, *S. typhimurium* LT2, *P. luminescens*), the nucleotide sequence of HI4320 was
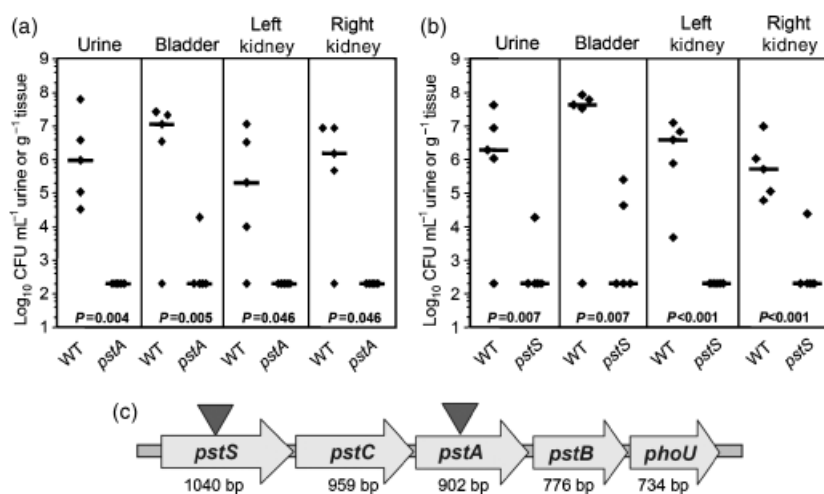

Fig. 1. *In vivo* competition between *pst* mutants and wild-type (WT) *Proteus mirabilis* strain HI4320. CBA mice were transurethrally co-inoculated with WT and either (a) G1-43 (*pstA::Tn5* or *pstA*, previously misidentified as *pstC*) or (b) H4-34 (*pstS::Tn5* or *pstS*). After 7 days, urine, bladders and kidneys were harvested to determine bacterial concentrations. Each data point represents the log 10 CFU mL^{-1} urine or g^{-1} tissue collected from one mouse. The horizontal bars represent the median. The limit of detection of this assay is 102 CFU mL^{-1} urine or g^{-1} tissue. Statistically significant differences in colonization were determined using a repeated measures analysis of variance with rank order data (STATA software). Significant *P* values are bolded ($P < 0.05$). WT *P. mirabilis* strain HI4320. (c) Schematic of the *pst* operon of *P. mirabilis* HI4320. Dark triangles represent the two *pst* genes that have been disrupted by Tn5 transposon mutagenesis.

Table 3. Characteristics of the *pst* operon of *Proteus mirabilis*

Gene	MW (kDa)	pI	Cellular Location	Probable Function
pstS	36 976	8.71	Periplasm	Periplasmic phosphate binding protein Domain from amino acid residues 27 to 319
pstC	34 634	5.27	Inner membrane	Inner membrane permease Predicted 6 transmembrane domains
pstA	33 087	9.95	Inner membrane	Inner membrane permease Predicted 6 transmembrane domains
pstB	29 118	6.34	Multiple sites	ATPase Domain from amino acid residues 36 to 230
phoU	27 883	5.28	Cytoplasm	Negative regulator of *pho* regulon

analyzed as described in 'Materials and methods'. Homologues of the *pho* regulon were identified in the *P. mirabilis* HI4320 genome and encoded proteins such as AP, PhoA, the two-component regulatory system PhoBR, three proteins associated with the assimilation and transport of phosphonates, PhnA, PhnP, and PhnX, and the putative ATPase of PhoH/PsiH (data not shown). In addition, *P. mirabilis* HI4320 possesses homologues of the low-affinity phosphate transporter, PitA, and the enzymes required for the production of and breakdown of polyphosphate, Ppk polyphosphate kinase, and Ppx/GppA exopolyphosphatase, respectively. All of these genes are known to be involved in phosphate assimilation and transport but are not known members of the *pho* regulon (Wanner, 1996; Ishige *et al.*, 2003). Notable homologues that were not detected by genome sequence analysis include the outer membrane porin PhoE and the low-affinity phosphate transporter PitB.

A general growth defect is not responsible for attenuation of the *pst* mutants *in vivo*

Before *in vivo* cochallenge studies, growth rates between *pst* mutants and wild-type *P. mirabilis* were performed individually and in coculture to determine whether *pst* mutants displayed altered growth rates *in vitro*.

For the individual growth studies in Luria broth, phosphate-limiting minimal salts medium, or pooled human urine, no statistically significant differences were observed in growth rates (recorded as change in $OD_{600\,nm}$) between the *pst* mutants and the wild-type strain over 48 h (data not shown). To corroborate the results observed in individual cultures, a more sensitive test, *in vitro* competition, was utilized to compare the growth of wild-type and *pst* mutants. For the most part, there were no significant differences between the amount of wild-type and *pst* mutant bacteria recovered during coculture in Luria broth or phosphate-limiting minimal salts medium; however, significant differences were observed for the *pstS*::Tn5 mutant in Luria broth at 48 and 72 h, and for the *pstA*::Tn5 mutant in phosphate-limiting minimal salts medium at 72 and 96 h (Fig. 2). At these times, there were *c.* 1–2-log differences in the amount of *pst* mutant present as compared with wild type (Fig. 2b and c). However, these results do not account for the dramatic decreases in recovery observed for the *pst* mutants (up to 6-logs) as compared with wild type during the 7-day *in vivo* cochallenge (Fig. 1).

To better mimic the nutritional environment of the urinary tract, additional *in vitro* competition studies were conducted using pooled human urine as a growth medium. Previous *in vitro* competition studies using urine as a growth medium have produced contrasting results, likely due to the association of *P. mirabilis* HI4320 with rapidly forming crystals, which in turn hampers the isolation of individual colonies and the ability to obtain accurate viable cell counts. Therefore, to better differentiate between the bacterial strains and crystalline structures, enumeration of the coculture of the wild-type and mutant bacteria grown in pooled
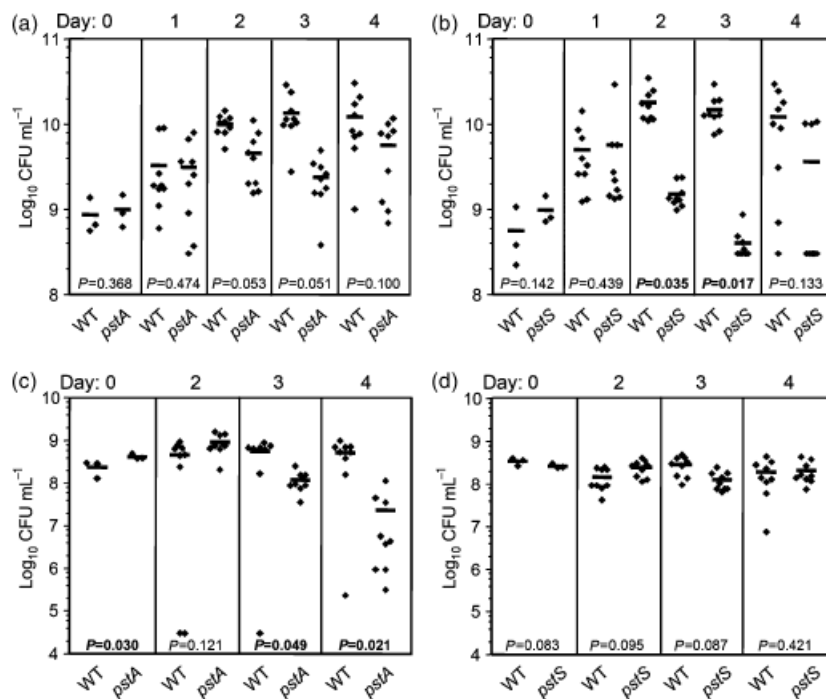

Fig. 2. *In vitro* competition of wild type (WT) and either (a,c) *pstA::Tn5* (*pstA*) or (b,d) *pstS::Tn5* (*pstS*) grown in (a,b) Luria broth and (c,d) phosphate-limiting medium. The horizontal bars represent the mean. Statistically significant differences in bacterial concentrations were determined using a Student's *t*-test. Significant values are bolded ($P < 0.05$). WT *Proteus mirabilis* strain HI4320.

human urine was accomplished through differential staining and imaging via confocal laser scanning microscopy (CLSM). In particular, all bacteria (wild-type and *pst* mutants) within the coculture were stained with Cell Tracker Orange CMRA, while only wild type was transformed with a stable plasmid, pBAC001 (Jansen *et al.*, 2003), which allowed for expression of the GFP in order to track wild-type bacteria. Despite being able to maintain close competition after one day of growth in pooled human urine, micrographs of the coculture surprisingly revealed that both *pst* mutants were able to outcompete the GFP-producing wild-type strain after 4 days of passage (Fig. 3, $P = 0.0002$, $P = 0.007$ respectively). Collectively, these results suggest that a severe growth defect cannot account for the attenuation observed in the mouse model because the *pst* mutants were recoverable after 4 days of passage.
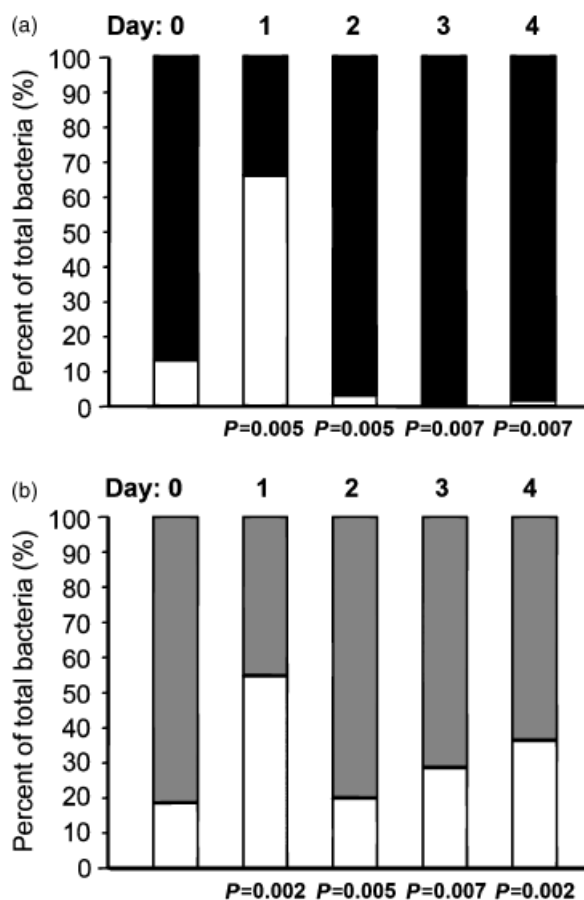

Fig. 3. *In vitro* competition assay of *Proteus mirabilis* HI4320 and *pst* mutants grown in pooled human urine as examined by confocal microscopy. (a) *In vitro* competition assay comparing *pstA::Tn5* mutant to the wild-type strain. (b) *In vitro* competition assay comparing *pstS::Tn5* mutant to the wild-type strain. The white bars represent the percent of total wild type (GFP-producing) bacteria and the dark black or gray bars depict the percent of total *pst* mutant bacteria. Statistical analysis was performed using a Student's *t*-test. Significant values are bolded ($P < 0.05$).

pst mutants constitutively synthesize AP

Previous studies have shown that *pst* mutants in *E. coli* and other bacterial species express *pho* regulon genes constitutively, including AP, regardless of phosphate levels (Wanner, 1986). As such, assays that measure the activity levels of this enzyme *in vitro* have been utilized as an indicator of the state of *pho* regulon and can thus be used to differentiate between these mutant strains and the wild type during growth in different media.

As expected, constitutive levels of AP activity were observed for the *pst* mutants as compared with wild type during growth in different media using an AP assay. When cultured in phosphate-rich Luria broth, both the *pstS*::Tn5 and *pstA*::Tn5 mutants constitutively expressed significantly higher levels of AP at all time points examined than the wild-type strain (Fig. 4a). Activity of the *pstS*::Tn5 and *pstA*::Tn5 mutants peaked at 24 h (1932 U, $P = 0.0005$) and 2 h (2095 U, $P < 0.0001$), respectively, compared with the wild type (peak at 5 h, 4.5 U) (Fig. 4a). AP activity ranged from 211 to 1932 U for the *pstS*::Tn5 mutant, 211–2095 U for the *pstA*::Tn5 mutant vs. 0.4–4.5 U for the wild type (Fig. 4a).

When cultured in phosphate-limiting minimal salts medium, both *pst* mutants continually expressed more AP than when cultured in Luria broth (Fig. 4b). Also, the mutants express more AP than the wild-type at the early time points (ranging as 1366 to 1560 U for *pstS*::Tn5 mutant, 915 to 1390 U for *pstA*::Tn5 mutant, and 0 U for the wild-type) (Fig. 4b). However, after 20 h, AP levels increased in the wild-type strain and remained at these higher levels, comparable to that observed in the *pst* mutant indicating induction of the *pho* regulon genes in *P. mirabilis* HI4320 when phosphate was depleted (peaked at 44 494 for the wild type, 53 197 for the *pstS*::Tn5 mutant, and 52 021 for the *pstA*::Tn5 mutant) (Fig. 4b). These results suggest that the *pho* regulon of the *pst* mutants is capable of further induction beyond what is described as constitutive expression by a mechanism that is not readily apparent.

When cultured in pooled human urine, both *pst* mutants constitutively expressed significantly more AP than wild type at all time points (Fig. 4c). However, AP levels were higher for the wild-type strain in pooled human urine than when cultured in Luria broth, but not as high as those observed for the strains grown in phosphate-limiting medium. The elevated presence of AP activity by *P. mirabilis* HI4320 during growth in human urine suggests that the genes of the *pho* regulon, including the Pst transporter, are expressed. Therefore, the Pst system is utilized during growth in this environment and thus may play a role during the infectious process in the urinary tract. To conclude, the AP assay is an effective and reliable method to differentiate the *pst* mutants of *P. mirabilis* HI4230 from the
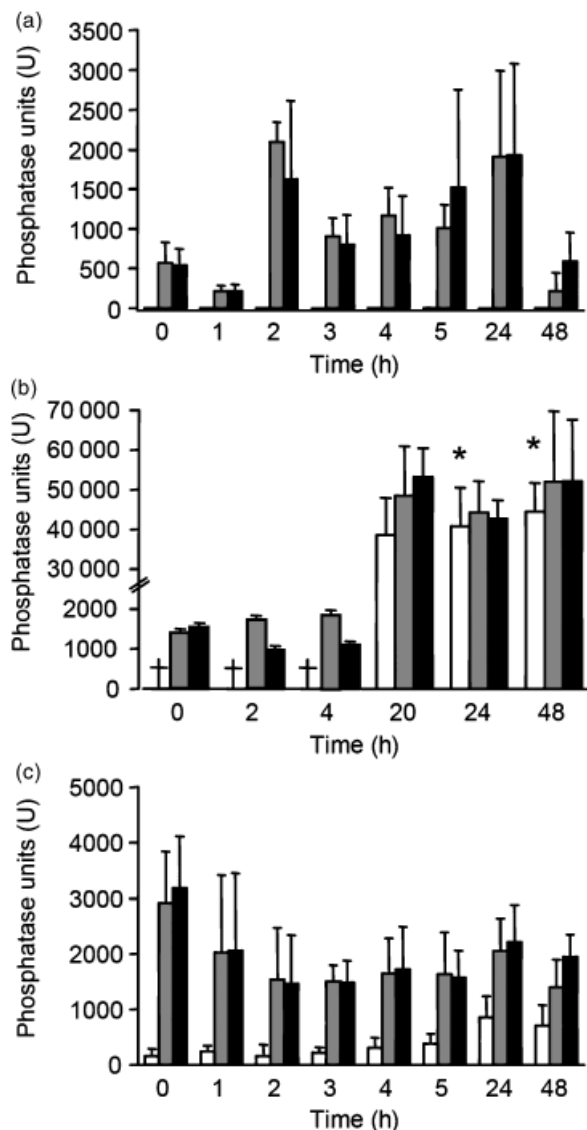
Fig. 4. Alkaline phosphatase activity of wild-type and the *pst* mutants grown in (a) Luria broth, (b) phosphate-limiting medium, and (c) pooled human urine. Phosphatase activity (in units) was plotted against time (hours postinoculation) for HI4320 wild type (white), *pstA::Tn5* (gray) and *pstS::Tn5* (black) mutants. Phosphatase units were calculated by the following equation: $[1000 \times OD_{405nm} - 1.75 \times (OD_{550nm})]/[\text{reaction time (min)} \times \text{volume (mL)} \times OD_{600nm}]$. The reaction time is the length of time (min) between the addition of the substrate *p*-nitrophenyl phosphate (*p*-NPP, 0.4%) and the stop solution K_2HPO_4 (1 M). Statistical analysis was performed using a Student's *t*-test. Unless indicated by an asterisk, the level of alkaline phosphatase activity was significantly greater ($P < 0.05$) for the *pst* mutants than for wild type. *$P > 0.05$. The wild-type strain exhibited no detectable alkaline phosphatase activity as indicated by crosses. The error bars represent the standard deviation.

wild-type strain when grown in different media. Further-more, this assay can be used to evaluate the state of the *pho* regulon in *P. mirabilis* HI4320 under different growth conditions.

In vitro complementation of the *pst* mutants

To establish that the Pst system plays a role in the virulence of *P. mirabilis* UTIs, mutations in the Pst system must be complemented. Transposon insertions in the *pstS* and *pstA* genes of the *pst* operon obviously disrupt the transcription of the gene containing the insertion. However, the transcription of genes downstream of the transposon in this operon may also be disrupted if the entire *pstSCAB-phoU* operon is transcribed as a single transcript as in other bacteria (Aguena *et al.*, 2002). Therefore, it was reasoned that the mutants in the Pst system in *P. mirabilis* must be comple-mented with the entire operon to restore the wild-type phenotype, namely, repression of the *phoA* gene in phos-phate-rich conditions and restoration of virulence in the CBA mouse model.

The *pst* mutants were complemented by PCR-amplifying the entire *pst* operon sequence from the *P. mirabilis* HI4320 chromosome and ligating the PCR fragment into the multi-ple cloning site of low-copy number plasmid pKHKS403. Clones possessing either vector alone or the entire *pst* operon were selected for ampicillin resistance and screened using a rapid AP assay for clones with restored repression of AP activity. The presence of the appropriate construct in each strain was confirmed and the transformed *pst* mutants were analyzed for *in vitro* complementation. In addition, we verified that complementation of the mutants with each disrupted gene alone (*pstS* or *pstA*) did not restore wild-type phenotype (Fig. 5), thereby lending support to conclusions in previous studies with other bacterial species that the *pst* operon is transcribed as a single transcript (Aguena *et al.*, 2002).

Transformation with the intact *pst* operon restored the wild type *in vitro* phenotype in the *pst* mutants. Comple-mentation of the *pstA::Tn5* mutant with the entire *pst* operon resulted in complete repression of AP activity (0 U) when cultured in the high phosphate-containing medium Luria broth at 17 h (Fig. 5), comparable to that observed for the wild-type strain (0 U). Significant reduction in AP levels was also observed in the *pstS::Tn5* mutant transformed with the *pst* operon [465 U as compared with *pstS::Tn5*: 1002 U ($P = 0.0008$)]. As expected, transformation of the *pst* mutants with either the vector alone [*pstS::Tn5* (pKHKS403): 820 U ($P = 0.197$); *pstA::Tn5* (pKHKS403): 732 U ($P = 0.073$)] or the intact *pst* gene insert [*pstS::Tn5* (403*pstS*): 869 U ($P = 0.456$); *pstA::Tn5* (403*pstA*): 684 U ($P = 0.106$)] did not result in a significant change in the levels of AP.

Satisfaction of molecular Koch's postulates for *pst in vivo*

Pst mutants of *P. mirabilis* HI4320 were severely attenuated for colonization of the urinary tract of the CBA mouse
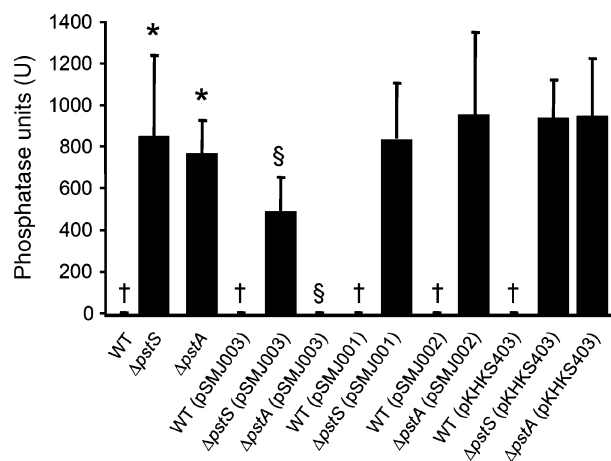
Fig. 5. *In vitro* complementation of the *pst* mutants as determined using the alkaline phosphatase assay. Phosphatase activity was examined after 17 h of growth in Luria broth. Each wild-type strain (WT), transformed with or without different complementing plasmids or vector alone (as indicated in parentheses) exhibited no detectable alkaline phosphatase activity (indicated by crosses). Only D*pstA* (*pstA::Tn5*) complemented with the entire *pst* operon (within pSMJ003) completely repressed alkaline phosphatase activity to WT levels. All other complemented *pst* mutants showed decreased or similar alkaline phosphatase activity to that seen in the *pst* mutants. Statistical analysis was performed using a Student's *t*-test. *$P < 0.05$ (WT vs *pst* mutant); §$P < 0.05$ (*pst* mutant vs complemented *pst* mutant). The error bars represent the standard deviation. WT *Proteus mirabilis* strain HI4320, Δ*pstS* (*pstS::Tn5*).
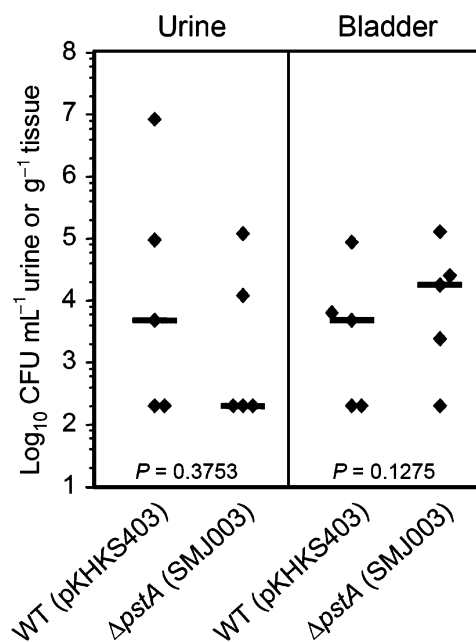

Fig. 6. *In vivo* complementation of *pstA* mutation. CBA mice were transurethrally co-inoculated with a 1 : 1 mixture of wild type (WT) containing the empty vector pKHKS403 and D*pstA* (*pstA::Tn5*) containing the complementing plasmid, pSMJ003. After 2 days, urine and bladders were harvested to determine bacterial concentrations. Each data point represents the Log 10 CFU mL^{-1} urine or g^{-1} tissue collected from one mouse. The horizontal bars represent the median. The limit of detection of this assay is 102 CFU mL^{-1} urine or g^{-1} tissue. Statistical differences in colonization were tested for using a repeated measures analysis of variance with rank order data (STATA software). $P > 0.05$ is not considered significant. WT *Proteus mirabilis* strain HI4320.

model as compared with the wild-type HI4320 strain (Fig. 1). To establish a role for the Pst system in virulence of *P. mirabilis* in UTI, complemented mutants in this transport system must regain their ability to colonize the urinary tract to numbers of CFU observed for the wild type. Because the wild-type phenotype was restored *in vitro* to the *pstA*::Tn5 mutant transformed with the *pst* operon, *in vivo* studies in the CBA mouse model of UTI were completed using the complemented *pstA*::Tn5 mutant.

These studies were accomplished in an *in vivo* cochallenge competition study comparing the ability of the wild-type HI4320 strain transformed with the pKHKS403 vector and the *pstA*::Tn5 mutant transformed with pSMJ003 (carries the entire *pst* operon) to colonize the urinary tract of the CBA mouse. After 2 days, the *pstA*::Tn5 mutant complemented with the complete *pst* operon was able to successfully compete and colonize the bladder in numbers similar (4.84×10^3 CFU g^{-1} bladder) to that observed for the wild type (1.79×10^4 CFU g^{-1} bladder; $P = 0.128$) and was present in urine at levels (2.00×10^2 CFU mL^{-1}) that were not significantly different from wild type (4.80×10^3 CFU mL^{-1}; $P = 0.375$), demonstrating *in vivo* complementation (Fig. 6). Therefore, molecular Koch's postulates for *pst* were satisfied for *pst* as a virulence factor in experimental UTI.

Discussion

Proteus mirabilis is commonly associated with complicated infections of the urinary tract. To understand the pathogenesis for this bacterial species, previous mutagenesis studies of the wild-type strain HI4320 in the CBA mouse model of ascending UTIs were extended to identify novel virulence factors. Here, evidence that *pstSCAB-phoU* is expressed as an operon in *P. mirabilis* is provided. Mutation of genes encoding the high-affinity Pst phosphate transporter results in the constitutive synthesis of AP from the loss of repression by PhoBR. While these mutants are not less fit than wild-type when cultured *in vitro*, they are severely attenuated in the murine model of UTI. Complementation of the mutants restores virulence and thus satisfies molecular Koch's postulates, establishing the Pst system as a virulence factor in *P. mirabilis*.

Sequence analysis revealed that the gene organization of the *pst* operon in *P. mirabilis* HI4320 is nearly identical to *E. coli* and other uropathogens (Amemura *et al.*, 1985; Kusaka *et al.*, 1997). Five potential Pst homologues of *P. mirabilis* were predicted to possess structural domains and critical

residues similar to those seen with *E. coli* and other enterobacteria. Therefore, it is not surprising that these homologues perform their respective functions as their counterparts in *E. coli*. Also, the results indicate that *P. mirabilis* HI4320 possesses a functional *pho* regulon because homologs associated with phosphate assimilation and transport (*phoBR, phnA, phnP, phnX, phoA, pitA, ppk, ppx/gppA, psiH, ugpCEABQ*) (Akiyama *et al.*, 1992; Kim *et al.*, 1993; VanBogelen *et al.*, 1996) appear to be present within the HI4320 genome.

The *pstS*::Tn5 and *pstA*::Tn5 mutants were previously shown to be recovered in significantly reduced numbers ($\geq 10^3$-fold fewer CFU mL^{-1} of urine or g^{-1} bladder) in the *in vivo* competition studies with wild type during ascending UTI. Independent *in vitro* growth studies, however, demonstrated that these mutants had no significant growth defect as compared with the wild type. To more fully evaluate whether the mutations in the *pst* operon produced a measurable growth defect, more sensitive competition culture studies were performed in Luria broth, phosphate-limiting minimal salts medium, and human urine. Although significant decreases in growth were noted for the *pst* mutants at certain time points and in certain growth conditions during competition culture studies against the wild-type strain (Fig. 2), these mutants were overall recoverable after 4 days of passage, suggesting that a severe growth defect is unlikely to account for the attenuation observed in the CBA mouse model.

The *pst* operon of *P. mirabilis* HI4320, as with the *pst* operon (*pstSCAB-phoU*) and other members of the *phosphate* regulon of *E. coli*, including AP, is regulated by the two-component regulatory system PhoBR and is repressed until times of phosphate starvation. Based upon previous studies in *E. coli* and other *pst* operon mutants (Willsky *et al.*, 1973; Rosenberg *et al.*, 1977; Amemura *et al.*, 1982; Kusaka *et al.*, 1997), it was hypothesized that this normal suppression would be relieved in the *P. mirabilis pstS*::Tn5 and *pstA*::Tn5 mutants, thereby resulting in constitutively produced AP due to the loss of repression by PhoBR as compared with the wild-type strain regardless of growth conditions. In experiments using Luria broth, phosphate-limiting minimal medium, and human urine, the level of AP was measured in wild-type *P. mirabilis* HI4320 and mutants in this operon in order to develop a rapid identification for these mutants. The AP levels were highly elevated under all culture conditions in the mutants compared with the wild type.

However, in the course of the study, there was a marked contrast in AP activity of the *pst* mutants that was dependent upon the growth medium utilized. Differences in AP activity were noted at 24 and 48 h postinoculation when the *pst* mutants were grown in phosphate-limiting medium (peaked *c*. 53 000 U) as compared with Luria broth with excess phosphate (peaked *c*. 1900 U) or pooled human urine

(peaked *c*. 2200 U), respectively (Fig. 4b and c). The disparity in AP activity observed was not due to the amount of bacterial growth, as the OD$_{600\,nm}$ measurements were not significantly altered (data not shown). Furthermore, wild-type HI4320 exhibited comparable AP activity levels when grown in phosphate-limiting medium at these time points (peaked *c*. 45 000 U), and therefore cannot be explained by induction of the *pho* regulon by the Pst system alone. Because the Pst system is not functional in the *pst* mutants because of genetic inactivation by transposon insertion, other mechanisms must be responsible for the additional induction of the *pho* regulon that was observed in the wild-type and mutant strains during growth in phosphate-limiting medium. In *E. coli*, it has been shown that the sensor CreC (formerly known as PhoM) induces the *pho* regulon in a PhoB-dependent manner but independent of phosphate concentration (Makino *et al.*, 1984). Currently, it is not known what stimuli are responsible for the induction of the *pho* regulon by CreC. Moreover, it has been demonstrated in *E. coli* that the *pho* regulon can be induced in a PhoB-dependent manner independently of phosphate concentration and PhoR through two components of carbon metabolism, phosphate acetyltransferase Pta and acetate kinase AckA (Wanner & Wilmes-Riesenberg, 1992). These enzymes are involved in a pathway that subsequently produces the metabolic intermediate acetyl phosphate that incorporates phosphate into ATP. Furthermore, it has been observed that PhoB is autophosphorylated *in vitro* in the presence of acetyl phosphate (Hiratsu *et al.*, 1995). Potential homologues of these enzymes have been discovered in *P. mirabilis* HI4320 (*pta*: PMI1772; *ackA*: PMI1771, data not shown), suggesting that the further induction of the *pho* regulon observed in the wild-type and *pst* mutants during growth in phosphate-limiting medium could be due to a Pst-independent mechanism that has not been previously characterized in *P. mirabilis*. Because phosphate is essential for many facets of metabolism, these alternative mechanisms of regulating phosphate metabolism provide a critical link between the genes involved in phosphate metabolism and central metabolism, thereby allowing cross talk and global control to be possible.

In a similar set of studies, an induction of AP activity by the wild-type HI4320 strain during growth in pooled human urine was observed. These data suggest that the *pho* regulon, including the Pst system, is induced and is therefore predicted to be expressed during UTI. Although the composition of human urine is not identical to that of murine urine, these media are comparable, and hence expression of PhoA and the members of the *pho* regulon are predicted to be expressed during growth in murine urine and during UTI. Taken together, the AP assay was an effective and reliable method to differentiate the *pst* mutants of *P. mirabilis* HI4230 from the wild-type strain.

The AP assay was also used in subsequent studies to test for complementation of the *pst* mutants when transformed with the *pstSCAB-phoU* operon to restore wild-type levels of *phoA* gene repression in phosphate-rich conditions. Results demonstrated that *in vitro* complementation or repression of AP levels of the *pst* mutants was accomplished in whole or in part by the introduction of the *pst* operon. While the complemented *pstA*::Tn5 mutant achieved full restoration of *in vitro* wild-type phenotype, the complemented *pstS*::Tn5 mutant achieved partial but nevertheless statistically significant restoration to wild-type AP activity. The incomplete complementation observed in the *pstS*::Tn5 mutant after the introduction of the entire *pst* operon may be due to the presence of truncated versions of the PstS protein generated by the insertion of the transposon competing with the full-length PstS for binding sites on the Pst permease. Previous work has shown that the *pstS* gene is located on the major transcript of the *pst* operon (Aguena *et al.*, 2002) and, as such, the promoter of the *pst* operon in the complementing vector may not be expressing enough PstS to fully complement the mutant strain and thus would explain the partial complementation observed in the *pstS* mutant.

The level of *in vivo* complementation was also determined in a cochallenge competition experiment comparing the ability of the wild-type HI4320 strain transformed with the empty pKHKS403 vector and the *pstA*::Tn5 mutant transformed with the cloned intact *pst* operon to colonize the urinary tract of the CBA mouse. In particular, it was observed that the *pstA*::Tn5 mutant transformed with the intact *pst* operon was able to compete and colonize the bladder in numbers similar to those observed for the wild type transformed with vector alone. Because complementation of the *pstA*::Tn5 mutant with the *pst* operon led to a restored recovery of the mutant during *in vivo* competition with wild-type, these data suggest that the Pst system plays an important role in the colonization and survival of *P. mirabilis* within the murine urinary tract during infection.

Because these *pst* mutants were generated by transposon mutagenesis, the phenotypes observed by these mutants may be due to polar effects of transposon insertion on downstream gene expression. Because neither cloned *pstA* nor *pstS* could fully complement its respective mutant, this indicates that the transposon mutants were likely polar. The use of the *pst* transposon mutants is nevertheless justified for this investigation because the focus of this study was to examine how the Pst system, not individual genes within the operon, is involved in the virulence associated with *P. mirabilis* UTIs.

Recently, there have been indications that the Pst transport system plays a role in biofilm formation, which has been shown to be critical for the establishment of chronic infections. Studies using null mutants in *pstC* and *pstA* genes revealed that the Pst system negatively regulates biofilm formation in *Pseudomonas aureofaciens* PA147-2 (Monds *et al.*, 2001). However, in the plant pathogen *Agrobacterium tumefaciens*, there is an increase in biofilm formation during phosphate limitation regulated by PhoBR (Danhorn *et al.*, 2004). Because biofilm formation is critical for the establishment of *P. mirabilis* in the human host including the encrustation of urinary catheters (Morris *et al.*, 1997), mutations in the Pst system of HI4320 could cause defects in this process, and therefore could explain the attenuation observed in the mouse model.

Lastly, mutagenesis studies have elucidated different potential roles for the Pst transport system because of the pleiotropic effects observed for mutants of this system. As previously stated, the Pst transport system has been demonstrated to play a role in pathogenesis, including roles in the regulation of biofilm formation (Monds *et al.*, 2001), invasion (Sinai & Bavoil, 1993; Lucas *et al.*, 2000; Mathew *et al.*, 2001), antibiotic resistance (Soualhine *et al.*, 2005), and colonization (Daigle *et al.*, 1995; Orihuela *et al.*, 2001; Lamarche *et al.*, 2005; Peirs *et al.*, 2005; Runyen-Janecky *et al.*, 2005; Buckles *et al.*, 2006). Disruptions due to mutations in the Pst system in any of these mechanisms or any infection processes unique to *P. mirabilis* should be sufficient to affect the fitness of this organism and subsequently cause the attenuation observed in the CBA mouse. Only further studies can determine if this is the case.

Acknowledgements

Authors' contribution

M.E.S. and H.L.T.M. contributed equally to this work.

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