Identification of protease and *rpoN*-associated genes of uropathogenic *Proteus mirabilis* by negative selection in a mouse model of ascending urinary tract infection

Hui Zhao,¹ Xin Li,¹ David E. Johnson² and Harry L. T. Mobley¹

Author for correspondence: Harry L. T. Mobley. Tel: +1 410 706 0466. Fax: +1 410 706 6751.
e-mail: hmobley@umaryland.edu

Department of Microbiology and Immunology¹ and Department of Veterans Affairs Medical Center², University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, USA

***Proteus mirabilis*, a motile Gram-negative bacterium, is a principal cause of urinary tract infections in patients with functional or anatomical abnormalities of the urinary tract or those with urinary catheters in place. Thus far, virulence factors including urease, flagella, haemolysin, various fimbriae, IgA protease and a deaminase have been characterized based on the phenotypic traits conferred by these proteins. In this study, an attempt was made to identify new virulence genes of *P. mirabilis* that may not have identifiable phenotypes using the recently described technique of signature-tagged mutagenesis. A pool of chromosomal transposon mutants was made through conjugation and kanamycin/tetracycline selection; random insertion was confirmed by Southern blotting of chromosomal DNA isolated from 16 mutants using the *aphA* gene as a probe. From the total pool, 2.3% (9/397) auxotrophic mutants and 3.5% (14/397) swarming mutants were identified by screening on minimal salts agar and Luria agar plates, respectively. Thirty per cent of the mutants, found to have either no tag or an unamplifiable tag, were removed from the input pool. Then 10⁷ c.f.u. from a 96-mutant pool (~10⁵ c.f.u. of each mutant) were used as an input pool to transurethrally inoculate seven CBA mice. After 2 d infection, bacteria were recovered from the bladders and kidneys and yielded about 10⁵ c.f.u. as an output pool. Dot blot analysis showed that two of the 96 mutants, designated B2 and B5, could not be hybridized by signature tags amplified from the bladder output pool. Interrupted genes from these two mutants were cloned and sequenced. The interrupted gene in B2 predicts a polypeptide of 37.3 kDa that shares amino acid similarity with a putative protease or collagenase precursor. The gene in B5 predicts a polypeptide of 32.6 kDa that is very similar to that encoded by ORF284 of the *rpoN* operon controlling expression of nitrogen-regulated genes from several bacterial species. The virulence of the two mutants was tested further by co-challenging CBA mice with each mutant and the parental strain. After 1 week of infection, the B2 and B5 mutants were recovered in numbers 100-fold and 1000-fold less than the parental strain, respectively. Using an *in vitro* assay, it was shown that the B2 mutant had significantly less (*P* = 0.0001) extracellular protease activity than the wild-type strain. These findings demonstrate that signature-tagged mutagenesis is a viable approach to identify bacterial genes associated with the ability to infect the urinary tract.**

Keywords: urinary tract infection, signature-tagged mutagenesis (STM), *Proteus mirabilis*, CBA mouse

Abbreviations: MR/P, mannose-resistant/*Proteus*-like; UTI, urinary tract infection.

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INTRODUCTION

Proteus mirabilis, a common uropathogen, is frequently isolated from the urine of hospitalized patients and individuals with indwelling urinary catheters. Infection with this organism can result in serious complications including acute pyelonephritis, stone formation in the bladder and kidney, encrustation and obstruction of the catheter, fever and bacteraemia (Griffith *et al.*, 1976; Mobley & Warren, 1987; Rubin *et al.*, 1986). After *Escherichia coli*, *P. mirabilis* is the second leading cause of bacteraemia in the elderly (Setia *et al.*, 1984).

Thus far, several virulence determinants including urease (Griffith *et al.*, 1976; Mobley & Warren, 1987; Jones *et al.*, 1990; Johnson *et al.*, 1993b), fimbriae (for review see Mobley & Belas, 1995), flagella (Belas & Flaherty, 1994; Allison *et al.*, 1994; Mobley *et al.*, 1996), haemolysin (Swihart & Welch, 1990; Mobley *et al.*, 1991), IgA protease (Senior *et al.*, 1987, 1991; Loomes *et al.*, 1990; Wassif *et al.*, 1995) and amino acid deaminase (Massad *et al.*, 1995) have been described for this species. The genes encoding these proteins have been isolated and sequenced and some of the corresponding proteins have been purified and characterized. To determine the contribution to pathogenesis of each virulence determinant, isogenic mutants have been constructed for most of these genes and tested for virulence in a CBA mouse model of ascending urinary tract infection (UTI). By these systematic studies, urease, flagella and mannose-resistant/*Proteus*-like (MR/P) fimbria have been found to contribute most significantly to the pathogenesis of acute pyelonephritis. *P. mirabilis* fimbriae (PMF) and haemolysin also contribute to virulence but appear to play more subtle roles (Massad *et al.*, 1994; Mobley, 1996).

Thus far, we have identified genes, suggested to play a role in virulence, that have a clear and measurable phenotype. To identify and assess the function of additional virulence factors for which phenotypes may not easily be recognized by plate assays, signature-tagged mutagenesis, a newly described genetic approach (Hensel *et al.*, 1995), was used in this study. In this technique, transposon mutagenesis is used to make chromosomal mutations. However, the transposon has been modified by adding unique 80 bp tags which can be identified by PCR and colony blot or dot blot hybridization. A mixed population of mutants, as an input pool, are inoculated into an appropriate animal model. After a defined period of infection, animals are killed and mutants with attenuated virulence (those not recovered) can be identified by hybridizing the tags from the recovered pool and comparing those present in the input pool. The genes interrupted by a transposon are cloned and sequenced. The function of the genes may be predicted by a sequence similarity search with a database. This system was developed and has been used successfully in *Salmonella typhimurium* and *Staphylococcus aureus* by D. W. Holden and colleagues (Hensel *et al.*, 1995; Shea *et al.*, 1996; Mei *et al.*, 1997) and others (Schwan *et al.*, 1998) and in *Vibrio cholerae* (Chiang &

Mekalanos, 1998), typically in models where high concentrations of organisms are recovered from tissue.

Reliable selection of mutants using signature-tagged mutagenesis is heavily dependent on the animal model. Since ascending infection is thought to be the most frequent route of human UTI, a well-established mouse model of ascending UTI (Hagberg *et al.*, 1983; Johnson *et al.*, 1987) was used in our study. This mouse model has been successfully used by our group to assess the uropathogenicity of *E. coli* (Johnson *et al.*, 1993a), *Providencia stuartii* (Johnson *et al.*, 1987) and *P. mirabilis* (Johnson *et al.*, 1993b). In this report, we describe the use of signature-tagged mutagenesis for the isolation of newly identified virulence genes of uropathogenic *P. mirabilis* in the CBA mouse model of ascending UTI. While numbers of bacteria recovered from the bladders of infected mice in these studies did not reach levels observed for other bacterial species in other animal models (Hensel *et al.*, 1995; Shea *et al.*, 1996; Mei *et al.*, 1997; Schwan *et al.*, 1998), the technique nevertheless appears useful for identification of virulence genes.

METHODS

Bacterial strains, plasmids and growth media. *P. mirabilis* HI4320 (urease-positive; produces MR/P, PMF and ambient temperature fimbriae; haemolysin-positive; Tet^R) was isolated from an elderly woman with urinary-catheter-associated bacteriuria (Mobley & Warren, 1987; Warren *et al.*, 1982). *E. coli* strain DH5 α λ pir was used to maintain the tagged pUT/mini-Tn5 plasmid pool and *E. coli* S17 λ pir was used as a donor strain for conjugation with *P. mirabilis* HI4320 (recipient strain). *E. coli* DH5 α [*supE44* Δ lacU169(ϕ 80lacZ Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used in cloning DNA. Luria broth (l⁻¹: 10 g tryptone, 5 g yeast extract and 5 g NaCl) and Luria agar (Luria broth containing 1.5% agar) were used as culture media. Non-swarming agar (l⁻¹: 10 g tryptone, 5 g yeast extract, 5 ml glycerol, 0.4 g NaCl and 29 g agar) was used to prevent swarming of *P. mirabilis* (Belas *et al.*, 1991a). Minimal salts medium for *Proteus* sp. contained (l⁻¹) 10.5 g K₂HPO₄, 4.5 g KH₂PO₄, 0.47 g sodium citrate and 1.0 g (NH₄)₂SO₄; after autoclave sterilization, 1 ml 1 M MgSO₄, 10 ml 20% (v/v) glycerol and 1 ml 1% (w/v) nicotinic acid were added to 1 l medium prior to pouring. Nitrogen-limited medium (Hu *et al.*, 1990) consisted of ammonium-free M9 medium (containing, l⁻¹, 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 mM MgSO₄, 0.4% glucose, pH 7.4) and 10 mM freshly prepared filter-sterilized L-glutamine or L-arginine. Nitrogen-excessive medium contained ammonium-free M9 medium and NH₄Cl (40, 400 and 800 mM).

Signature-tagged mutagenesis. Plasmid DNA representing the tagged pool (pUT/mini-Tn5 marked with random signature tags; Amp^R) (Hensel *et al.*, 1995) was transformed into donor strain *E. coli* S17 λ pir by electroporation. Transformants were pooled and stored in Luria broth with 15% (w/v) glycerol at -70 °C. Recipient cells of *P. mirabilis* HI4320 (Tet^R) were grown overnight at 37 °C in Luria broth containing tetracycline (20 μ g ml⁻¹). Overnight culture (50 μ l) was inoculated into 5 ml fresh Luria broth and grown to OD₆₀₀ 0.5–1.0; a large loopful of frozen donor cells of S17 λ pir harbouring the tagged pUT/mini-Tn5 plasmid was inoculated

into 3.5 ml Luria broth and grown for 1.5 h to allow recovery of bacteria. In a 96-well microtitre plate, 50 µl of each donor and recipient cells was mixed and a 48-well metal replicator was used to transfer samples of the strain suspensions from the microtitre dishes onto the non-swarming Luria agar plate. After overnight mating and growth, each mating suspension was restreaked onto non-swarming Luria agar plates containing kanamycin (50 µg ml⁻¹) and tetracycline (20 µg ml⁻¹). A single colony from each mating was selected at random and stored in a single microtitre plate well.

Southern blot analysis. Chromosomal DNA isolated from *P. mirabilis* transposon mutants was digested with restriction enzymes and electrophoresed on a 0.8% (w/v) agarose gel, and transferred to a membrane (QIABRANE Nylon Plus; Qiagen). Probe labelling, hybridization and signal detection were carried out with the ECL direct nucleic acid labelling and detection system (Amersham Life Science) according to the instructions of the manufacturer.

Colony blot and dot blot hybridization. For colony blots, bacteria were grown directly on sterile nylon membranes overlaid onto dry non-swarming LB agar plates containing kanamycin (50 µg ml⁻¹). Plates were incubated right side up overnight at 37 °C. Filters were prepared for hybridization as instructed by the manufacturer (Qiagen). DNA probes were labelled with [³²P]dCTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); Amersham] by hot PCR and hybridized to membranes under stringent conditions (50% formamide, washing at 65 °C). For dot blots, bacteria were lysed and DNA was denatured in 0.6 M NaCl, 0.2 M NaOH and 0.08% SDS for 10 min or more prior to blotting onto the membrane. Membranes were washed in 2 × SSC (1 × SSC contains, l⁻¹, 8.765 g NaCl, 4.41 g sodium citrate, pH 7.0) and hybridized with probes under stringent conditions (50% formamide, washing at 65 °C).

Measurement of dot blot signals. The integrated density of each colony's dot blot signal from the autoradiographs was measured using the Eagle Eye II Still Video System (The Eagle Sight Software, version 3.0; Stratagene).

PCR. Transposon mutants (ranged in number from 10² to 10⁵), grown on non-swarming agar plates, were collected using disposable inoculating loops and suspended in 50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20 containing proteinase K (200 µg ml⁻¹). Samples were incubated for 1 h at 55 °C followed by incubation at 95 °C for 10 min to inactivate the protease. A sample of the supernatant, typically 1–10 µl in 100 µl PCR mixture, was used for amplification (Wright & Manos, 1990). For PCR, *Taq* DNA polymerase was used according to the directions of the manufacturer. After an initial denaturation of 4 min at 95 °C, the thermocycler was programmed for 20 cycles of 50 °C for 45 s, 72 °C for 10 s and 95 °C for 30 s. Primers P2 (5' TACCTACAACCTCAAGCT 3') and P4 (5' TACCCATTCTAACCAAGC 3') were synthesized on an Applied Biosystems Automated DNA Synthesizer. The first PCR was performed in 100 µl volumes. After ethanol precipitation, PCR products were purified by electrophoresis through a 1.6% (w/v) Seaplaque agarose (FMC Bioproducts) gel in TAE buffer. Gel slices containing fragments of about 80 bp were excised and a 2 µl sample was used for the second PCR. This reaction was carried out in a total volume of 20 µl and contained 50 µM each of dATP, dTTP and dGTP and 10 µl [³²P]dCTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); Amersham]. The second PCR products were digested with *Hind*III to remove primer arms and used directly for hybridization (Hensel *et al.*, 1995).

Molecular cloning and nucleotide sequencing. Chromosomal DNA was isolated from transposon mutants and digested with

several restriction enzymes, including *Eco*RI, *Sma*I, *Pvu*II, *Nsi*I, *Dra*I and *Ssp*I. Digests were electrophoresed through agarose gels and subjected to Southern blot analysis (as described above) using the kanamycin-resistance gene of pUT/mini-Tn5-Kan (1.7 kb *Eco*RI-*Xba*I fragment) as a probe. Restriction enzymes which gave rise to hybridizing fragments in the range 2.5–10 kb were selected for digestion of DNA for cloning. Digested fragments were ligated into *Eco*RV-digested pBluescript KS(–) and transformed into *E. coli* DH5α. Transformants were selected on LB plates containing kanamycin (50 µg ml⁻¹). Sequencing was performed by the dideoxy chain-termination method with double-stranded DNA as the template. Reagents from the PRISM Ready Reaction DyeDeoxy Termination kit (Applied Biosystems) were used in conjunction with *Taq* polymerase (Boehringer Mannheim). Reactions were run on a model 373 DNA sequencer (Applied Biosystems).

CBA mouse model of ascending UTI. A modification (Johnson *et al.*, 1987) of the mouse model of ascending UTI originally developed by Hagberg *et al.* (1983) was used. Female mice (20–22 g, 6–8 weeks old; Jackson Laboratory, Bar Harbour, ME, USA), tested for the absence of bacteriuria, were anaesthetized with methoxyflurane and inoculated with *P. mirabilis* HI4320 [approx. 10⁷ c.f.u. suspended in 0.05 ml PBS (l⁻¹: 8 g NaCl, 0.2 g KCl, 11.5 g Na₂HPO₄·7H₂O, 2 g KH₂PO₄); 10⁵ c.f.u. each of the 96 mutants] through a sterile polyethylene catheter inserted into the bladder through the urethra. After 1 week, the mice were killed by administration of an overdose of methoxyflurane. Urine was collected and the bladder and both kidneys were removed. Each sample was quantitatively cultured, and viable counts were determined as c.f.u. (ml urine)⁻¹ or c.f.u. (g tissue)⁻¹.

Proteolytic assay. A single colony of *P. mirabilis* HI4320 or its signature-tagged mutant was inoculated into Luria broth (5 ml) and cultured overnight with shaking at 37 °C. The bacterial culture was diluted 1:50 into 100 ml fresh Luria broth and incubated at 37 °C with shaking until the OD₆₀₀ reached approximately 1.0. For the supernatant fraction, 1 ml cell culture was added to microcentrifuge tubes and subjected to centrifugation (10000 g, 5 min, 4 °C). Supernatants were collected and held on ice for assay on the same day. For periplasmic fractions, bacterial pellets from 3 ml cultures were treated with 2 mg polymyxin B ml⁻¹ at 37 °C for 30 min and the supernatants were separated from the pellet by centrifugation (10000 g, 5 min, 4 °C), collected and held on ice. The remaining overnight cultures were centrifuged (7000 g, 10 min, 4 °C) and the cell pellets were resuspended in 1.5 ml Luria broth and lysed in a French pressure cell at 20000 p.s.i. (138 MPa). After centrifugation (27000 g, 20 min, 4 °C), supernatants were collected and held on ice.

Triplicate samples from supernatant, periplasmic and cytoplasmic fractions (300 µl) were added separately to microcentrifuge tubes containing 500 µl 2% azocasein and 0.2% sodium azide in 50 mM HEPES buffer (pH 7.5). Tubes were incubated overnight at 37 °C. Trichloroacetic acid (30% as final concentration) was added to precipitate undigested azocasein. After microcentrifugation, the A₄₀₀ of the supernatant from each tube was measured. Data are expressed as A₄₀₀ (ml original culture volume)⁻¹. Means of triplicate experiments were compared using the *t*-test.

Other assays. The γ-glutamyl transferase assay (Bender *et al.*, 1977) is used to measure the total amount of glutamine synthetase activity (adenylated plus non-adenylated enzyme). Urease activity was measured quantitatively by the phenol red spectrophotometric method of Hamilton-Miller &

Gargan (1977) as described previously (Jones & Mobley, 1987). MR/P haemagglutination was demonstrated by the agglutination of untreated chicken erythrocytes (Bahrani & Mobley, 1994; Bahrani *et al.*, 1994).

RESULTS

Preparation of the mutant pool

A pool of mini-Tn5 transposon with signature tags was introduced into *P. mirabilis* chromosomal DNA by conjugation between *E. coli* S17 λ pir (tagged pUT/mini-Tn5-Kan) as donor and Tet^R *P. mirabilis* HI4320 as recipient. Exconjugants were selected on non-swarming Luria agar plates containing kanamycin (50 μ g ml⁻¹) and tetracycline (20 μ g ml⁻¹). All Kan^R Tet^R colonies were further screened for ampicillin susceptibility since some suicide plasmid pUT/mini-Tn5 might still be retained as a replicating plasmid in *P. mirabilis*. By screening on Luria agar plates containing ampicillin (100 μ g ml⁻¹) and tetracycline (20 μ g ml⁻¹), only a very few colonies were found to be Amp^R; these Amp^R colonies were excluded from the mutant bank. Of 397 mutants examined, another 30% of colonies, with either no tag or an unamplifiable tag, were identified by colony blot hybridization and also removed from the mutant

pool. Thus approximately 280 colonies met criteria for inclusion.

To confirm that the transposon inserted into different sites within the chromosome of individual mutants, Southern hybridization was done using the 1.7 kb *Eco*RI-*Xba*I fragment (Lorenzo *et al.*, 1990) carrying the kanamycin-resistance gene from plasmid pUT/mini-Tn5-Kan as a probe. Chromosomal DNA was isolated from 16 randomly picked mutants and digested with *Xba*I, which has a unique restriction site inside the transposon (Fig. 1). None of the 16 mutants had comparable digestion patterns, suggesting that the insertions were random for the pool of transposon mutants.

As another index of random insertion, we screened for mutants that were incapable of swarming and for auxotrophic mutants by plating the mutants both on freshly prepared Luria agar and on minimal salts agar, respectively. We found approximately 3.5% swarming mutants (i.e. did not swarm) and 2.3% auxotrophic mutants (i.e. no growth on minimal medium). According to previous reports, at least 45 genes from the *P. mirabilis* genome are involved in directing motility, cell differentiation and multicellular behaviour, an important process for bacteria to colonize and ascend the urinary tract (Allison & Hughes, 1991; Belas *et al.*, 1991b); about 70 genes, when mutated, lead to auxotrophy (Belas *et al.*, 1991a). The frequency of these mutations further suggested that the mutations were random and evenly distributed around the *P. mirabilis* chromosome (based on a genome size of 4.2 Mb; Allison & Hughes, 1991).

Experimental animal infection and dot blot analysis

We used the CBA mouse model of ascending UTI, which mimics the natural route of the human infection (i.e. ascension of bacteria from the urethra to the bladder to the ureter to the kidney). Using 96 mutants as an input pool, we conducted animal infection studies for different periods of time and recovered the output pools from six mice after 2 d infection and six mice after 7 d infection. We found that infection for 2 d gave more reproducible results for bladder samples than 7 d infection; results from kidney samples have not been as reproducible (about 25% of the mutants are not recovered).

Two days after challenge, mice were killed and bacteria were recovered by plating bladder or kidney homogenates onto non-swarming Luria agar plates supplemented with kanamycin and tetracycline. The geometric means of log₁₀ c.f.u. (ml urine)⁻¹ or log₁₀ c.f.u. (g bladder or kidney)⁻¹ were 8.86 for urine, 5.87 for bladder and 4.07 for kidney. These results are typical of experimental infection using this method (Bahrani & Mobley, 1994; Jones *et al.*, 1990; Mobley *et al.*, 1996). Approximately 10⁵ c.f.u., recovered from each bladder, were used as an output pool.

An example of a dot blot analysis of an input pool and an output pool from the bladder of one experimentally

Fig. 1. Southern blot of randomly selected transposon mutants. Chromosomal DNA was prepared from 16 mutants randomly chosen from the pool of 96 mutants, using plasmid DNA with a tagged transposon as a positive control and the chromosomal DNA from the wild-type *P. mirabilis* as a negative control. All DNA samples were digested with *Xba*I, which has a unique restriction site inside the transposon. A 1.7 kb *Eco*RI-*Xba*I fragment carrying the kanamycin-resistance gene from the tagged plasmid was used as a probe by the ECL direct labelling system.

Fig. 2. Dot blot hybridization analysis from (a) the input pool and (b) the output pool from a bladder of an infected mouse. A pool of 96 mutants, including two auxotrophic mutants (E12 and F12) and two swarming mutants (G12 and H12), was inoculated into CBA mice. Two days after inoculation, bacteria were recovered from the bladders and represented the output pool. DNA isolated from approximately 10^4 c.f.u. of the input pool or output pool was used as template for PCR amplification. ^{32}P -labelled PCR products (80 bp fragments containing pooled signature tags), digested with *HindIII*, were used as probes for hybridization of the dot blot membranes prepared from the 96 mutants.

Table 1. Densitometric analysis of autoradiographs of dot blots from the output and input pools of *P. mirabilis* signature-tagged transposon mutants inoculated into and recovered from the bladders of transurethraly challenged CBA mice

Each number represents the quantitative densitometric signal for the dot blot hybridization of the output pool (organisms recovered from the bladders of five mice following 2 d infection) divided by the densitometric signal for the dot blot hybridization of the input pool (organisms used to transurethraly challenge the mice). Mutants with ratios of <0.15 (underlined; B2 and B5) were selected for analysis. For each of the five mice the $\log_{10}$ c.f.u. (g bladder) $^{-1}$ recovered 48 h after inoculation was 6.691, 6.589, 7.078, 4.566 and 7.223.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.26	1.10	1.10	2.50	0.77	0.50	0.38	0.80	0.59	0.24	1.25	0.69
B	0.75	<u>0.13</u>	0.74	0.61	<u>0.04</u>	0.69	0.72	0.86	0.73	0.94	0.87	0.75
C	0.60	0.76	1.24	0.41	0.25	0.30	0.33	0.27	1.11	>10	0.97	1.48
D	1.18	0.42	1.67	0.34	0.94	1.09	0.92	0.83	0.46	0.54	0.91	1.15
E	0.78	1.00	1.18	0.77	0.73	0.80	0.85	0.74	0.77	1.16	0.65	0.44
F	>10	1.32	0.90	0.68	1.88	0.94	5.50	0.28	0.52	1.21	1.06	0.54
G	0.77	0.78	0.81	0.75	1.03	0.97	0.79	0.78	0.91	1.13	0.90	0.65
H	1.08	1.43	0.73	0.22	0.70	0.33	0.20	0.59	0.29	0.75	0.32	0.87

infected mouse is shown in Fig. 2. Since the colony signals from six output pools were not absolutely reproducible, the integrated density of each colony's dot blot signal from the autoradiographs derived from the

input pool and the output pool from five mice was assessed by a computerized gel documentation system. Data were pooled and mean densities (Table 1) for bladder samples from five mice revealed that two

Fig. 3. Assessment of the virulence of (a) B2 and (b) B5 mutants in the CBA mouse model of ascending UTI. Mice were co-challenged with each mutant and the *P. mirabilis* parental strain. After 1 week of infection, the mice were killed and c.f.u. (ml urine)⁻¹ or (g tissue)⁻¹ of the mutant and wild-type strain were determined on kanamycin/tetracycline or tetracycline Luria agar plates, respectively. Horizontal lines represent the geometric mean of the colony counts. P values were derived by using an unpaired t-test. For the B2 mutant, $P > 0.05$; for the B5 mutant, P values are shown above the figure. WT, Wild-type strain *P. mirabilis* HI4320; L, left; R, right.

mutants were reproducibly unrecoverable. These mutants, designated B2 and B5, were identified as those with attenuated virulence. Surprisingly, neither the auxotrophic mutants (E12, F12) nor the swarming mutants (G12, H12) included in this input pool were significantly attenuated.

Confirmation of attenuation by experimental infection

Of 96 transposon mutants tested, two mutants, B2 and B5, were consistently not recovered or recovered in negligible numbers. To confirm the attenuated virulence of these mutants, mice were transurethrally co-challenged with 5×10^6 c.f.u. each (total 10^7 c.f.u. ml⁻¹) of one mutant and wild-type strain. After 1 week, mice were killed and the mutant and wild-type strain were recovered and enumerated on Luria agar containing kanamycin and tetracycline (detects mutant only) or Luria agar containing only tetracycline (detects mutant plus wild-type), respectively. Log_{10} c.f.u. (ml urine)⁻¹ and (g bladder/kidney)⁻¹ were determined (wild-type vs mutant) for the B2 mutant ($n = 5$) (urine, 9.92 vs 7.51; bladder, 7.62 vs 5.60; and kidneys, 5.70 vs 3.83; $P > 0.05$) and for the B5 mutant ($n = 5$) (urine, 9.86 vs 1.52, $P = 0.001$; bladder, 6.43 vs 2.19, $P = 0.02$; and kidneys, 4.52 vs 1.13, $P = 0.05$) (Fig. 3).

Identification of interrupted genes

To identify the nature of the attenuated transposon mutants, the interrupted genes were cloned from the B2 and B5 mutants and sequenced. Chromosomal DNA was isolated from each mutant and digested separately with *EcoRV*, *SmaI*, *PvuII* and *DraI* (all are predicted to cut within the transposon). Using the kanamycin-resistance gene from transposon Tn5 as probe, Southern blot hybridization showed that the B2 mutant, digested with *EcoRV*, produced a ~10 kb hybridizing fragment and the B5 mutant digested with *DraI* produced a 2.6 kb hybridizing fragment (data not shown). Therefore, *EcoRV*-digested B2 chromosomal DNA and *DraI*-digested B5 chromosomal DNA were used for ligation into the *EcoRV*-digested pBluescript KS(-) vector. Transformants were selected on Luria agar plates containing kanamycin. The DNA fragments interrupted with the Mini-Tn5 transposon were confirmed as being cloned by digestion with several restriction enzymes. Both of the chromosomal DNA fragments flanking the transposon were subcloned into pBluescript KS(-) and sequenced using T3 and T7 promoter sequences from the pBluescript vector as primers. ORFs were identified using Genepro (version 5.00; Riverside Scientific) and each of the putative ORFs was used to search for similar sequences in the NR (non-redundant) GenBank using BLAST (Table 2).

Sequence analysis and characterization of the B2 mutation

From B2 mutant subclones we determined 2.3 kb of sequence data which includes sequence (530 bp) upstream of the B2 ORF, the entire B2 ORF (1002 bp) and part of the sequence from a second ORF (793 bp) downstream of the B2 gene (GenBank accession no. AF088981). The first ORF which was interrupted by the transposon (after nucleotide 561 of this ORF) predicts a polypeptide of 37.3 kDa. The two ORFs are 14 bp apart

Table 2. Homologues of *P. mirabilis* genes identified as virulence factors by signature-tagged mutagenesis

Interrupted gene	Product of the homologous gene	Strain	Accession no.	P*
B2	Putative protease	<i>Escherichia coli</i>	P45527	1.9e-194
		<i>Haemophilus influenzae</i>	P44700	1.7e-22
	Collagenase	<i>Escherichia coli</i>	D90846	9.8e-19
		<i>Methanococcus jannaschii</i>	B64311	3.5e-28
		<i>Porphyromonas gingivalis</i>	P33437	1.7e-10
B5	ORF284 in the <i>rpoN</i> operon	<i>Escherichia coli</i>	P33995	2.8e-53
		<i>Klebsiella pneumoniae</i>	P17163	2.8e-53
		<i>Haemophilus influenzae</i>	P45071	3.3e-36
		<i>Bacillus subtilis</i>	Z94043	8.3e-13

* P, Smallest sum probability. It contains the lowest P value ascribed to any set of high-scoring segment pairs for each database sequence.

Table 3. Proteolytic activity of *P. mirabilis* and a signature-tagged mutant

Values are the mean and standard deviation of protease activity [proteolytic activity A_{440} (ml cell culture)⁻¹] detected by the azocasein protease assay (see Methods).

Strain	Fraction		
	Supernatant	Periplasmic	Cytoplasmic
Wild-type	0.511 ± 0.029	0.032 ± 0.016	0.040 ± 0.004
B2 mutant	0.301 ± 0.040*	0.011 ± 0.001*	0.023 ± 0.002*

* Significantly lower than wild-type: supernatant, $P < 0.0001$; periplasmic fraction, $P = 0.0115$; cytoplasmic fraction, $P < 0.0001$.

and each of them has a ribosome-binding site upstream of their start codon. Although the promoter has not been identified, it is very likely that both ORFs would use the same promoter found upstream of the B2 ORF. One hundred and ninety-eight base pairs upstream of the B2 ORF a 330 bp ORF is predicted to be transcribed in the opposite direction to that of B2. The second ORF was found to share amino acid similarity with products of genes of unknown function from *E. coli*.

By hydropathy analysis, the amino-terminal sequence (ELLCPAGNLPALKAAIENGADA) of the B2 protein is predicted to be relatively hydrophobic but does not fit to the classical signal peptide specifications. From the SWISS-PROT Sequence Bank (release 35.0, August, 1997), we found that the B2 gene and its homologues belong to a peptidase family called U32, the function of which is still not understood. In this family, only one gene, *prtC*, a protease gene from *Porphyromonas gingivalis*, has been well characterized. It was reported that *prtC* encoded a collagenase capable of cleaving type I collagen (Kato *et al.*, 1992).

To investigate the function and localization of the B2 protein, proteolytic activity of the wild-type strain and B2 mutant strain was tested using an azocasein proteolytic assay (Table 3). The B2 mutant had significantly

less ($P = 0.0001$) proteolytic activity than the wild-type strain, consistent with the B2 gene encoding a protease. Most of the protease activity partitioned with the extracellular fraction, suggesting that the protease is secreted.

Characterization of the B5 mutant

The gene interrupted in the B5 mutant (GenBank accession no. AF088980) is an 855 bp ORF predicting a polypeptide of 32.6 kDa which is highly similar to ORF284 in the *rpoN* operon of *E. coli* and *Klebsiella pneumoniae* (Jones *et al.*, 1994). We also have complete sequence from the B5 downstream gene, which appears to be very similar to *npr* of the *rpoN* operon (Fig. 4). The *rpoN* operon encodes RNA polymerase σ^{54} factor, which is required for expression of a wide variety of nitrogen-regulated genes including glutamine synthetase (*glnA*). Therefore, to determine the effect of the B5 mutant on σ^{54} activity, we assessed glutamine synthetase activity from wild-type and B5 mutant strains. Three ammonia concentrations were used: 1, 3 and 20 mM NH_4Cl in M9 minimal salts medium. In 3 and 20 mM NH_4Cl , we did not find any significant difference in the glutamine synthetase activity of the wild-type and B5 mutant. However, when only 1 mM NH_4Cl was used as sole

Fig. 4. Interrupted ORF in the B5 mutant and its homologue in the *E. coli* *rpoN* operon. Two ORFs were identified within 1297 nucleotides (black boxes). The first ORF predicts a polypeptide of 32.6 kDa and shares amino acid sequence identity with ORF284 of the *E. coli* *rpoN* operon. The GenBank accession number for this sequence is AF088980. The transposon insertion site (black triangle) and the DNA region that has been sequenced is shown between the two dotted lines. Percentages represent the amino acid sequence identity between the products of homologous genes.

nitrogen source, glutamine synthetase activity was reduced in the mutant as compared to the wild-type strain (data not shown).

To assess the possibility that the attenuated virulence of B5 was due to a defect in the expression of other putative virulence genes, urease activity and expression of MR/P fimbriae were evaluated. Using a urease assay (Jones & Mobley, 1988), we detected the same level of urease activity from the wild-type and from the B5 mutant. Using a haemagglutination assay, both the wild-type and the B5 mutant were tested for the ability to haemagglutinate chicken erythrocytes in the presence of mannose. We found that the B5 mutant can haemagglutinate as well as the wild-type, indicating that, at least *in vitro*, the mutation in the B5 gene would not affect MR/P fimbriae expression (Bahrani & Mobley, 1994; Zhao *et al.*, 1997).

We also wanted to find whether the attenuation of virulence was due to poor growth of the B5 mutant in urine. Since the urea concentration in urine is about 400–500 mM (Griffith *et al.*, 1976), urine can be classified as a very nitrogen rich medium. However, it is hard to assess growth in urine *in vitro* because *P. mirabilis* urease hydrolyses urea, elevates pH and causes ion precipitation. Therefore, we used M9 minimal medium containing different concentrations of nitrogen sources. Growth rates of the wild-type strain and the B5 mutant were determined in nitrogen-limited media (10 mM glutamine or arginine) and nitrogen-excessive media (400 mM and 800 mM NH_4Cl). We found that the growth rate of the B5 mutant was not altered with respect to the wild-type strain in nitrogen-limited media (10 mM glutamine or arginine) but was slightly (not significantly different) reduced in nitrogen-excessive media (400 mM NH_4Cl). Neither wild-type nor B5 mutant could grow in medium containing 800 mM NH_4Cl (data not shown).

DISCUSSION

In contrast to *in vivo* expression technology (Mahan *et al.*, 1993) which uses positive selection, signature-tagged mutagenesis is a genetic approach for identifying puta-

tive virulence genes by negative selection in animal models of infection. In our studies, using signature-tagged mutagenesis, two previously unidentified genes involved in virulence have been isolated and characterized. Mutations in these genes result in attenuation of *P. mirabilis* HI4320 in an experimental mouse model of UTI. Two days after transurethral challenge, a protease mutant was recovered in numbers 100-fold less than the parental strain. A mutant in what appears to be a σ^{54} operon was recovered in numbers over 1000-fold less than the parental strain from urine, bladder and kidneys. Since the function of these two mutated genes had not previously been recognized, signature-tagged mutagenesis has proven to be a viable approach to identify genes associated with the ability to infect the urinary tract.

Unlike the animal model from the *S. typhimurium* studies (Hensel *et al.*, 1995) which has very strong selective capabilities, our CBA mouse model of ascending UTI did not allow us to identify the attenuated mutants in every experiment. Some mutants in addition to the two reproducibly unrecoverable mutants were lost during individual experiments. This may have been due to the open route of the urinary tract where bacteria can easily be flushed out by urine flow. Therefore, as opposed to only two mice being necessary to identify *S. typhimurium* mutants, we had to challenge a higher number of mice ($n = 7$) and pool the results to identify a reproducible pattern. We also had to modulate the infection period from 7 d to 2 d, since 7 d infection, which has been used in most of our previous animal infection studies, may have eliminated many mutants with perhaps only slight reductions in virulence. In addition, it appeared more difficult to obtain reproducible results from kidneys; we found that even the blotting of mutants from two kidneys from the same mouse gave quite different results. This is undoubtedly a result of the lower numbers of organisms (usually a total of 10^4 c.f.u. g^{-1} or only a mean of $\sim 10^2$ c.f.u. for each mutant) that characteristically are recovered from an infected kidney.

The protease gene, identified through signature-tagged mutagenesis, appears to represent a virulence deter-

minant, since mutation of the gene attenuates virulence in the mouse model of ascending UTI. This gene is homologous with genes encoding putative proteases or collagenases and appears to encode a secreted product. Thus far, an IgA-degrading protease (ZapA) is the only protease that has been identified in *P. mirabilis* (Wassif *et al.*, 1995) and no virulence studies have yet been conducted using isogenic mutants. However, alignment of the respective predicted amino acid sequences demonstrates that there is no similarity between the B2 gene product and this IgA-degrading protease or to those of other mucosal pathogens including *Neisseria gonorrhoeae* (Plaut *et al.*, 1975) and *Haemophilus influenzae* (Plaut, 1983). Instead, the B2 gene encodes a protein that is related to the U32 peptidase family. In this family, only PrtC from *Porph. gingivalis* has been characterized as a collagenase. In their review, Häse & Finkelstein (1993) classified PrtC collagenase in the calcium-containing metalloprotease family. A spontaneous mutant of a *Porph. gingivalis* strain, which was attenuated for virulence in a rodent model system, was defective in several potential virulence characteristics including the collagenase activity (Shah *et al.*, 1989). These data suggested that collagenase could be a virulence factor. However, isogenic mutants have not been tested.

UTIs by *Proteus* spp. can result in severe histological damage, resulting from development of acute pyelonephritis, calculi formation and renal impairment (Mobley, 1996). This damage could partly be explained by the action of proteases. Extracellular bacterial proteases from a number of pathogenic organisms have been demonstrated to play important roles in virulence. By degrading host tissue substrates like collagen, fibronectin and proteinaceous receptors, these enzymes can destroy host mucosal or skin barriers (Häse & Finkelstein, 1993) and promote pathogen colonization (Kontani *et al.*, 1997). By degrading immunoglobulin or serum complement components, they may also avoid the host immune response (Häse & Finkelstein, 1993). By facilitating detachment of the pathogen from the initial niche, they can help infection to spread (Finkelstein *et al.*, 1992). Finally, by acting as toxins, they can enhance vascular permeability (Chowdhury *et al.*, 1991) or cause mucosal haemorrhage (Brunner *et al.*, 1997).

It is interesting that a secreted protease was identified by this method. One might expect that members of the input pool could complement the defect in the B2 mutant, i.e. protease secreted by the other 95 members of the input pool should be capable of supplying the protease missing from the B2 mutant. There is actually some evidence for what has been called 'soft attenuation'. When the B2 mutant is placed into direct competition with the wild-type strain (Fig. 3a), a dramatic attenuation is not observed as for the B5 mutant. Indeed, although the sample size is small (five mice in each group) and the *P* values approach levels of significance, the mutant when co-infected with the wild-type still displays some limited potential for colonization. It would be interesting to compare levels of

colonization in groups of mice challenged separately with either the mutant or the wild-type strain alone.

Another putative virulence gene is the B5 gene, which is very similar to ORF284 of the *rpoN* operon. The *rpoN* operon encodes σ^{54} factor for RNA polymerase (Jones *et al.*, 1994; Merrick & Coppard, 1989; Powell *et al.*, 1995). Many cellular functions depend on activation of transcription by a σ^{54} promoter, most of which are related to cellular nitrogen assimilation and nitrogen fixation; others include decarboxylate transport, pilin and flagellin synthesis and urea utilization (Merrick, 1993; Merrick & Edwards, 1995). Thus far, seven ORFs have been identified in the *rpoN* operon of *E. coli*. Since the two genes we identified are almost identical to ORF284 (92% amino acid sequence identity of predicted polypeptides) and *npr* (56% amino acid sequence identity) of the *rpoN* operon, we propose that *P. mirabilis* also has an *rpoN* operon. Two downstream ORFs, *ptsN* and *npr*, in the *rpoN* operon are homologous with the IIA enzyme (specific for fructose and mannitol transport) and HPr in the phosphoenolpyruvate-dependent phosphotransferase systems (PTS) (Jones *et al.*, 1994; Powell *et al.*, 1995). The primary function for the PTS system is for sugar reception, transport and phosphorylation. The secondary function is to mediate metabolic and transcriptional regulation (Saier & Reizer, 1994). Moreover, it was reported in *K. pneumoniae* that mutation in ORF95 and *ptsN* could elevate σ^{54} activity (Merrick & Coppard, 1989). Therefore, it has been suggested that the *rpoN* operon represents a link that may coordinate carbon and nitrogen metabolism in the bacterial cell (Jones *et al.*, 1994; Merrick, 1993) and these downstream ORFs might provide a means for sensing nutritional status and regulating σ^{54} activity by phosphorylation (Imaishi *et al.*, 1993; Reizer *et al.*, 1992). Since the B5 mutation does not significantly alter growth of *P. mirabilis* *in vitro*, it must exert its effect by altering expression of critical but as yet undetected genes that are expressed *in vivo*.

Based on this information, we have made an effort to investigate the contribution to virulence of the B5 gene. We have determined bacterial growth rate in nitrogen-limited and -sufficient media, tested the glutamine synthetase activity in nitrogen-limited and -sufficient media, and measured the expression of the other virulence factors including urease and MR/P. Unlike the σ^{54} -promoter-dependent urease gene in *Klebsiella* sp., urease activity in *P. mirabilis* is controlled by a urea-dependent AraC-like positive transcriptional activator (UreR) (Nicholson *et al.*, 1993) and thus this activity was not altered in the B5 mutant. MR/P fimbriae expression also did not change. Because urine is a nitrogen-sufficient and sugar-depleted medium, the mutation in ORF284 (B5) might affect bacterial growth in the urinary tract. However, we did not find any significant difference between the wild-type and B5 mutant of growth rate in nitrogen-limited, nitrogen-excessive and sugar-deficient M9 media. There was evidence that the synthesis of glutamine synthetase was reduced when NH_4Cl as sole nitrogen source was limited to 1 mM. Therefore, mu-

tation in B5 most likely affects expression of classic nitrogen-regulated genes and as yet undetected but critical nitrogen-regulated genes that play a key role in survival of the organism during UTI of the host.

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