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

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*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.

The GenBank accession numbers for the sequences in this paper are AF088980 and AF088981.
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, 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<sup>R</sup>) was isolated from an elderly woman with urinary-catheter-associated bacteriuria (Mobley & Warren, 1987; Warren et al., 1982). E. coli strain DH5α <sup>pir</sup> was used to maintain the tagged pUT mini-Tn<sub>5</sub> plasmid pool and E. coli S17 <sup>pir</sup> was used as a donor strain for conjugation with P. mirabilis HI4320 (recipient strain). E. coli DH5α [supE44 ΔlacU169(q80lacZAM15) hsdR1 recA1 endA1 gyrA96 thi-1 relA1] was used in cloning DNA. Luria broth (1:1:10 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 (1:1:10 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 (1:1:1) 10·5 g KH<sub>2</sub>PO<sub>4</sub>, 4·5 g KH<sub>2</sub>PO<sub>4</sub>, 0·47 g sodium citrate and 10·g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; after autoclave sterilization, 1 ml 1 M MgSO<sub>4</sub>, 10 ml 20% (w/v) glycerol and 1·1 ml 1% (w/v) nicotinic acid were added to 11 medium prior to pouring. Nitrogen-limited medium (Hu et al., 1990) consisted of ammonium-free M9 medium (containing, 1·1, 6·g Na<sub>2</sub>HPO<sub>4</sub>, 3·g KH<sub>2</sub>PO<sub>4</sub>, 0·5 g NaCl, 1 M MgSO<sub>4</sub>, 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 NH<sub>4</sub>Cl (40, 400 and 800 mM).

Signature-tagged mutagenesis. Plasmid DNA representing the tagged pool (pUT mini-Tn<sub>5</sub> marked with random signature tags; Amp<sup>R</sup>) (Hensel et al., 1995) was transformed into donor strain E. coli S17 <sup>pir</sup> 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<sup>R</sup>) were grown overnight at 37°C in Luria broth containing tetracycline (20 μg ml<sup>−1</sup>). Overnight culture (50 ml) was inoculated into 5 ml fresh Luria broth and grown to OD<sub>595</sub> 0·5–1·0; a large loopful of frozen donor cells of S17 <sup>pir</sup> harbouring the tagged pUT mini-Tn<sub>5</sub> 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 (QUIBRANE 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 on 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 M sodium citrate, pH 7.0) and hybridized with probes 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 1 l⁻¹, 8765 g NaCl, 441 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 prior to blotting onto the membrane. Membranes were overlaid onto dry non-swarming LB agar 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 Hageberg 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* H4320 [approx. 10⁹ 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); 10⁶ 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* H4320 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 OD₅₇₀ 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 A₆₀₀ of the supernatant from each tube was measured. Data are expressed as A₆₀₀ (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 zipr (tagged pUT/mini-Tn5-Kan) as donor and TetR P. mirabilis HI4320 as recipient. Exconjugants were selected on non-swarming Luria agar plates containing kanamycin (50 μg ml⁻¹) and tetracycline (20 μg ml⁻¹). All KanR TetR 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 μg ml⁻¹) and tetracycline (20 μg ml⁻¹), only a very few colonies were found to be AmpR; these AmpR 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 EcoRI-Xbal 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 XhoI, 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 10⁶ 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
Negative selection of *P. mirabilis* virulence genes

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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 transurethrally 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 transurethrally 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.

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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 transurethrally challenged CBA mice

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...
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 $5 \times 10^6$ 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, Smal, 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

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.
genes of unknown function from plasmic fraction, hundred and ninety-eight base pairs upstream of the B2 gene, opposite direction to that of B2. The second ORF was which is still not understood. In this family, only one By hydropathy analysis, the amino-terminal sequence predicted to be relatively hydrophobic but does not fit to gingivalis, belong to a peptidase family called U32, the function of identified, it is very likely that both ORFs would use the same promoter found upstream of the B2 ORF. One ORF a 330 bp ORF is predicted to be transcribed in 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 SWISS-PROT Sequence Bank (release 35.0, August, 1997), we found that the B2 gene and its homologues has been well characterized. It was reported that prtC, a protease gene from Porphyromonas gingivalis, has been well characterized. It was reported that prtC encoded a collag enase 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 nzp of the rpoN operon (Fig. 4). The rpoN operon encodes RNA polymerase \( \sigma^{54} \) 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 \( \sigma^{54} \) activity, we assessed glutamine synthetase activity from wild-type and B5 mutant strains. Three ammonia concentrations were used: 1, 3 and 20 mM \( \text{NH}_4\text{Cl} \) in M9 minimal salts medium. In 3 and 20 mM \( \text{NH}_4\text{Cl} \), we did not find any significant difference in the glutamine synthetase activity of the wild-type and B5 mutant. However, when only 1 mM \( \text{NH}_4\text{Cl} \) was used as sole 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 (ELLCPAGNLPAKLKAIENGADA) 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.

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

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

* \( P \), 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Supernatant</th>
<th>Periplasmic</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.511 ± 0.029</td>
<td>0.032 ± 0.016</td>
<td>0.040 ± 0.004</td>
</tr>
<tr>
<td>B2 mutant</td>
<td>0.301 ± 0.040*</td>
<td>0.011 ± 0.001*</td>
<td>0.023 ± 0.002*</td>
</tr>
</tbody>
</table>

* Significantly lower than wild-type: supernatant, \( P < 0.0001 \); periplasmic fraction, \( P = 0.0115 \); cytoplasmic fraction, \( P < 0.0001 \).
To assess the possibility that the attenuated virulence of strain (data not shown). reduced in the mutant as compared to the wild-type B5 was due to a defect in the expression of other putative glutinate as well as the wild-type, indicating that, at least in uitro, 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 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 uitro, the mutation in the B5 gene would not affect MR/P fimbriae expression (Bahrani & Mobley, 1994; Zhao et al., 1997).

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 10⁶ c.f.u. g⁻¹ or only a mean of ~ 10⁴ 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-

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**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.
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., 1993) 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 *Porphyromonas 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 *Porphyromonas 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 (Brunder 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 B3 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_5 factor for RNA polymerase (Jones et al., 1994; Merrick & Copbard, 1989; Powell et al., 1995). Many cellular functions depend on activation of transcription by a σ^54_5 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_5 activity (Merrick & Copbard, 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_5 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_5-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 fimbiae 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 NH₄Cl 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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Negative selection of *P. mirabilis* virulence genes


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