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Construction of a Urease-Negative Mutant of *Proteus mirabilis*: Analysis of Virulence in a Mouse Model of Ascending Urinary Tract Infection

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***Proteus mirabilis*, a urease-producing uropathogen, causes serious urinary tract infections in humans. To specifically evaluate the contribution of urease to virulence, a mutation was introduced into *P. mirabilis* HI4320 by homologous recombination. Virulence was assessed in the CBA mouse model of ascending urinary tract infection. Twenty mice each were challenged transurethrally with *P. mirabilis* HI4320 and its urease-negative derivative (1×10^9 to 2×10^9 CFU). At 48 h animals were sacrificed and the mean $\log_{10}$ CFU per milliliter of urine (parent, 6.23; mutant, 4.19; $P = 0.0014$) or per gram of bladder (parent, 6.29; mutant, 4.28; $P = 0.0002$), left kidney (parent, 4.11; mutant, 1.02; $P = 0.00009$), and right kidney (parent, 4.11; mutant, 2.43; $P = 0.036$) were all shown to be significantly different. These data demonstrate a role for urease as a critical virulence determinant for uropathogenic *P. mirabilis*.**

Urinary tract infection (UTI) with *Proteus mirabilis* may lead to serious complications such as fever, acute pyelonephritis, bacteremia, periurethral abscesses, catheter obstruction, renal stones, chronic inflammatory changes, and death (14, 17, 27, 29). Urease is recognized as a major virulence factor for *P. mirabilis* by virtue of its ability to rapidly generate ammonia from the hydrolysis of urea present in urine. An elevated pH results in ion precipitation in the form of struvite or carbonate-apatite kidney or bladder stones (11). Ammonia may also have a direct cytotoxic effect upon kidney cells in cultures (4).

To assess the contribution of urease in the development of *Proteus* pyelonephritis, a specific chromosomal mutation was introduced into urease gene sequences by homologous recombination. The urease-negative mutant and the parent strain were evaluated in a mouse model of ascending UTI. Our results indicate that urease plays a significant role in infection and can be characterized as an important virulence determinant for this species.

P. mirabilis HI4320 (Tet^r) is a human urinary tract isolate (24). *Escherichia coli* SY327 λ *pir* and *E. coli* SM10 λ *pir* (Kan^r) were obtained from John Mekalanos and have been described elsewhere (23). Plasmid pMDJ301, the source of the urease gene fragment, is an M13mp19 derivative containing a 1.5-kilobase *Hin*dIII fragment subcloned from the gene sequences of the *ureC* urease structural subunit of the *P. mirabilis* urease operon (16). Plasmid pGP704 (Ap^r), which was used as a suicide vector, was also obtained from John Mekalanos (23). *P. mirabilis* strains were maintained on Luria agar (21) containing 2% agar and 0.5% glycerol to prevent swarming (M. Robert Belas, personal communication).

Filter mating (28) was used to mobilize the suicide vector; cointegrates were selected on the appropriate antibiotic agar plates.

Mannose-resistant hemagglutination (mannose-resistant *Klebsiella*-like and mannose-resistant *Proteus*-like fimbriae) was assayed by the procedure of Old and Adegbola (26) and as described elsewhere (H. L. T. Mobley and G. R. Chippendale, J. Infect. Dis., in press).

The hemolytic titer was measured against sheep erythrocytes as previously described (Mobley and Chippendale, in press) and defined as the highest serial dilution in which the supernatant was visibly red due to hemoglobin release.

Chromosomal DNA was isolated from bacterial cells by the method of Marmur (22). Following restriction enzyme digestion and electrophoresis in a 0.7% agarose gel, DNA was transferred to nitrocellulose as described by Maniatis et al. (21). The urease gene probe, a 4.4-kilobase *Cla*I fragment from pMID1003 (15) spanning the majority of the urease operon, was labeled with [α-^{32}P]ATP (Dupont, NEN Research Products, Boston, Mass. [ca. 800 to 1,000 Ci/mmol]) by random primer extension (2). Hybridization was carried out under stringent conditions (50% formamide, 65°C wash). The blot was washed, dried, and autoradiographed.

A modification (13) of the mouse model of ascending UTI originally described by Hagberg et al. (12) was used for this study. Female CBA/J mice (Jackson Laboratory, Bar Harbor, Maine), 22 to 24 g, were used as the test animals. Urine specimens were cultured 24 h prechallenge, and mice with bacteria present at $>10^2$ CFU/ml were not used. The mice were each inoculated over a 30-s interval with 0.05 ml containing 2×10^{10} to 4×10^{10} CFU/ml through a sterile polyethylene catheter (0.28-mm internal diameter, 0.61-mm outer diameter, 25-mm length) which had been inserted into the bladder through the urethra. The mice were sacrificed with an overdose of methoxyflurane 48 h after challenge; urine samples, the bladder, and both kidneys of each mouse were removed aseptically, weighed, and separately quantitatively cultured by using a spiral plater (Spiral System Instruments, Inc., Bethesda, Md.). Viable counts were determined as CFU per milliliter of urine or gram of tissue.

The geometric means of the number of CFU per milliliter or gram were compared by the Student *t* test. Numbers of animals categorized by degree of colonization were compared by using the chi-square test.

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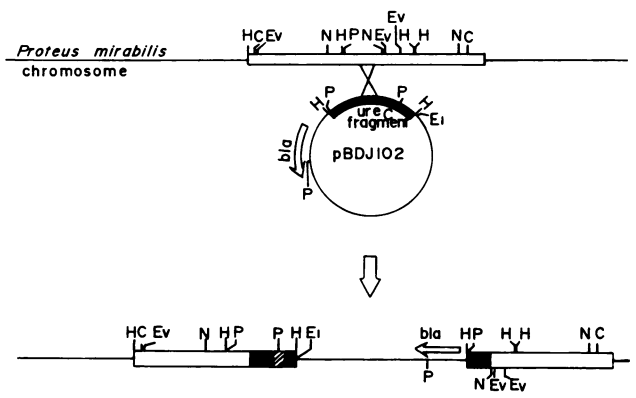

FIG. 1. Integration of the urease suicide plasmid into the chromosome of *P. mirabilis* HI4320 by homologous recombination. *E. coli* SM10 λ *pir* (Kan^r), carrying transfer genes integrated into its chromosome, was used to mobilize pBDJ102 (Ap^r) into *P. mirabilis* HI4320. Integration of pBDJ102 into the chromosome was selected on medium containing tetracycline and ampicillin. Transconjugants (Ap^r Tet^r) were urease negative. bla, β-lactamase; C, *Cla*I; Ei, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; N, *Nru*I; P, *Pvu*I. The 1.5-kilobase *Hin*dIII fragment of *ureC* (■), a 277-base-pair deletion (▨), and chromosomal urease gene sequences (□) are shown.

A suicide plasmid, pBDJ102, containing an *Eco*RI-*Pvu*II fragment (base pairs 1943 to 3447), was constructed in the vector pGP704 (23). Insertion of this plasmid into the chromosome of *P. mirabilis* (Tet^r) was accomplished by mobilization with *E. coli* SM10 λ *pir*(pBDJ102) (Ap^r) and selection on Luria plates containing tetracycline (20 μg/ml) and ampicillin (200 μg/ml). Analysis of ampicillin- and tetracycline-resistant transconjugants (two colonies of 10^9 organisms plated) revealed them to be urease-negative, ampicillin-resistant *P. mirabilis* isolates (Fig. 1). These strains and the parent strain had identical Minitek taxonomic identification numbers (BBL Microbiology Systems, Cockeysville, Md.), except for a negative urease test. To gain some idea of the crossover frequency for a urease-positive revertant, the cointegrate strain was passaged 10 times in the absence of ampicillin. No urease-positive colonies were recovered, even when $>10^7$ colonies were plated onto a single plate. Therefore, we estimated that the frequency of obtaining a urease-negative double crossover was less than 1 in 10^7 colonies.

Since we determined that the insertional mutation was sufficiently stable and that a reversion by a second crossover would not represent a problem in data analysis, we proceeded to characterize the cointegrate strain which carried a specific disruption of *ureC*, the gene encoding the largest of three urease structural subunits. Comparison of the parent strain *P. mirabilis* HI4320 with the urease mutant *P. mirabilis* HI4320 revealed that they had similar generation times of 33 and 29 min, respectively, in Luria broth at 37°C with aeration (200 rpm); identical hemolytic titers of 1:32 against a 5% sheep erythrocyte suspension; identical hemagglutination patterns characteristic of strains expressing both mannose-resistant *Klebsiella*-like and mannose-resistant *Proteus*-like hemagglutinins; and compatible Dienes swarming types (5) (Fig. 2). Southern blot analysis of chromosomal DNA revealed that the suicide vector had inserted into the chromosomal urease gene sequences of *ureC* between base pairs 1943 and 2929 (16). The only apparent difference between the parent and mutant strains was the production of urease (53.0 μmol of NH_3 per min per mg for the parent and <0.001 μmol of NH_3 per min per mg for the mutant).

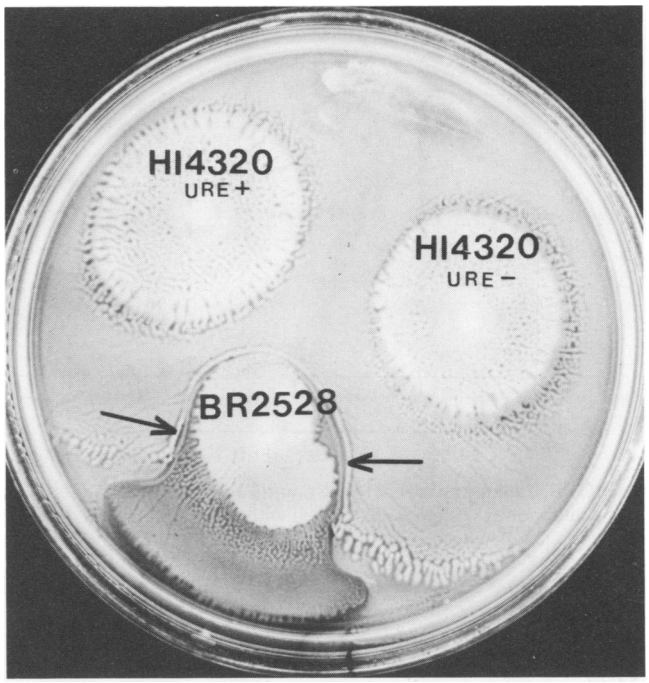

FIG. 2. Compatible swarming types of *P. mirabilis* HI4320 and urease-negative *P. mirabilis* HI4320, as determined by the Dienes phenomenon. *P. mirabilis* HI4320 (URE$^+$), its urease-negative derivative (URE$^-$), and strain BR2528 were spotted onto the Luria agar plate and allowed to swarm overnight. Compatible Dienes groups (5) have overlapping swarms, as demonstrated for strain HI4320 and its urease-negative derivative. A line of demarcation between strains of different Dienes groups, strains HI4320 and BR2528, is marked with arrows.

Twenty mice each were challenged transurethrally with urease-positive *P. mirabilis* HI4320 and urease-negative *P. mirabilis* HI4320. Bacteria from urine, the bladder, and the kidneys were quantitated 48 h after challenge. In no case was reversion from the urease-negative to the urease-positive phenotype observed. Analysis of the colony counts revealed that the urease mutant did not infect the urine, bladder, or kidneys as well as the uropathogenic parent strain (Fig. 3). Statistical analysis of the geometric mean of colony counts from urine samples, bladders, and kidneys of mice challenged with the parent or mutant revealed them to be significantly different (Table 1). Mice challenged with the parent strain had mean $\log_{10}$ CFU of 6.23/ml of urine and 6.29/g of bladder tissue, while mice challenged with the mutant had mean $\log_{10}$ CFU of 4.19/ml of urine and 4.28/g bladder tissue. The mean $\log_{10}$ CFU of parent bacterial counts in both the left and right kidneys was 4.11/g of tissue, while the mean $\log_{10}$ CFU of mutant strain counts in the left and right kidneys were 1.02 and 2.43/g of tissue, respectively.

The urease-negative mutant was apparently cleared more rapidly from the urinary tract than the parent strain. Only 1 of 20 mice challenged with urease-negative *P. mirabilis* HI4320 had $\geq 10^7$ CFU/ml in the urine, while 14 of 20 mice challenged with *P. mirabilis* HI4320 had $\geq 10^7$ CFU/ml in the urine ($P = 0.00009$). Of 20 mice challenged with the mutant, 2 had $\geq 10^6$ CFU/g in the bladder, in contrast to 16 of 20 mice for the parent strain ($P = 0.00004$). Of 39 kidneys from parent-challenged mice, 31 had $>10^3$ CFU/g, while only 15 of 40 had $>10^3$ for mutant-challenged mice ($P = 0.0004$).

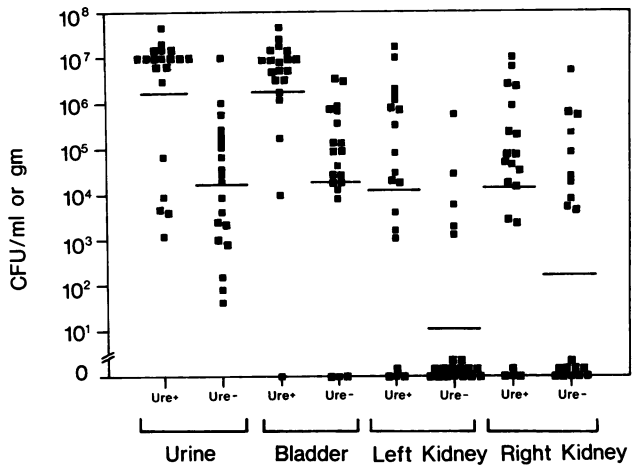

FIG. 3. Quantitative bacterial counts in urine specimens, bladders, and kidneys of mice challenged with either *P. mirabilis* HI4320 or its urease-negative derivative. Each square represents CFU per milliliter of urine or gram of tissue from an individual mouse. Horizontal lines represent the geometric mean of the colony counts. Squares on or near the *x* axis represent 0 CFU/ml or g.

Mice inoculated with the parent strain more often had organisms in both kidneys (14 of 19) than those inoculated with the mutant strain (2 of 20) ($P = 0.0002$). The number of animals with bacteria in neither kidney tended to be lower (3 of 20) for those inoculated with the parent strain than for those inoculated with the mutant (7 of 20) ($P = 0.27$). One stone was recovered from an animal infected with the parent strain; none were recovered from animals infected with the mutant strain.

Urease appears to play a highly significant role in the colonization of the urinary tract by *P. mirabilis* and might well be the critical virulence determinant required for the development of acute pyelonephritis by this species. The genetically constructed strain bearing a disruption of *ureC*, the chromosomal gene encoding the 61-kilodalton subunit of the native enzyme, is unable to synthesize an active urease. The lack of urease accounts for an astounding reduction of CFU per gram in the kidneys of mice 48 h after transurethral challenge. A geometric mean of 12,900 CFU/g of kidney tissue was obtained for 39 kidneys from 20 animals inoculated with the parent strain, in sharp contrast to only 50 CFU/g recovered from 40 kidneys of 20 mice inoculated in an identical fashion with the urease-negative mutant.

The inability to colonize the kidney severely limits the pathogenicity of this species, as the kidney appears to be the favored niche of *P. mirabilis* in the urinary tract (4). Bladder washout studies by Fairley and co-workers (6) demonstrated that *Proteus* species were localized primarily to the kidneys of humans with symptoms of acute UTI. Several investigators (4, 7, 10, 18, 19) have used rat or mouse models to compare the severity of pyelonephritis caused by *P. mirabilis*. The effects of urease were studied by injecting acetone-killed organisms that retained urease activity (4); by treating infected animals with oral supplements of acetohydroxamic acid, a specific inhibitor of the enzyme (1, 20, 25); or by inoculation of an ethyl methanesulfonate-generated urease mutant (18). Although all studies supported the supposition that urease played a role in virulence, the use of killed organisms (4), cytotoxic inhibitors (30), or mutants generated by nonspecific mutagens (18) makes interpretation difficult. Additionally, animals in these studies were challenged hematogenously, which establishes kidney infection by a route that is not now believed to mimic the more natural course of infection that develops by the ascending route.

TABLE 1. Quantitative cultures of urine, bladder, and kidney specimens from CBA mice challenged transurethrally with *P. mirabilis* HI4320 and its urease-negative derivative[a]

Strain	Mean $\log_{10}$ CFU/ml or g[b] of specimens from:			
	Urine	Bladder	Kidney	
			Left	Right
HI4320 (Ure^+)	6.23	6.29	4.11	4.11
HI4320 (Ure^-)	4.19	4.28	1.02	2.43
P value	0.0014	0.0002	0.00009	0.036

[a] Twenty mice were challenged with each strain.
[b] Per milliliter of urine or gram of tissue.

The contribution of urease to virulence of a gram-positive uropathogen has also recently been examined in *Staphylococcus saprophyticus* (8). A urease-negative *S. saprophyticus* mutant and the same strain carrying cloned urease genes were compared in an ascending unobstructed UTI rat model. In these studies urease contributed mainly to cystopathogenicity, that is, colonization and inflammation of the bladder.

The CBA mouse model of ascending UTI has previously been shown to be useful in studying bacterial virulence factors relevant in human UTI (12). Other models require manipulation and obstruction of the urinary tract (9) or introduction of inoculum by the hematogenous route of infection (3). The ascending route model was selected since it most nearly mimics the natural course of infection (27). In contrast, the initial event in models relying on the hematogenous route of infection is colonization of the kidney. It is not surprising that an avirulent organism introduced directly into the bloodstream could colonize and cause renal damage, while under natural conditions the organism might not persist in the urinary tract, much less ascend the ureters to the kidney.

Mutagenesis of the *P. mirabilis* chromosome by homologous recombination with cloned sequences represents, to the best of our knowledge, the first specific mutation introduced into this species by genetic methods. We attempted to introduce mutations in the chromosomal urease genes of *P. mirabilis* by delivering cloned urease genes carrying a mutation by using conjugative incompatible plasmids, suicide plasmids delivered in lambdoid particles, and spontaneous curing of multicopy plasmids carrying the mutated urease genes. No chromosomal insertions were isolated by any of these techniques. Use of the R6K *ori-pir* protein suicide system, however, yielded urease-negative cointegrates.

Our studies demonstrate that urease contributes significantly to the pathogenesis of pyelonephritis caused by *P. mirabilis*. These data do not rule out the contribution of other potential virulence determinants. Nearly all isolates of this species produce urease and produce about the same level of enzyme whether isolates are cultured from urine specimens or feces of patients with acute pyelonephritis (Mobley and Chippendale, in press). Therefore, urease production is not a trait unique to pathogenic isolates. Colonization factors, as yet undefined, or fimbriae (mannose-resistant *Proteus*-like or mannose-resistant *Klebsiella*-like) may bind specifically to kidney epithelium and allow ammonia, the product of urea hydrolysis, to be delivered at high concentrations to kidney tubular epithelium. It might be that this bacterium-host interaction is essential for pathogenesis.

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