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Construction of a Flagellum-Negative Mutant of *Proteus mirabilis*: Effect on Internalization by Human Renal Epithelial Cells and Virulence in a Mouse Model of Ascending Urinary Tract Infection†

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To examine the role of flagella in pathogenesis of urinary tract infection caused by *Proteus mirabilis*, we constructed a nonmotile, nonswarming flagellum mutant of strain WPM111 (an *hpmA* hemolysin mutant of strain BA6163, chosen because of its lack of in vitro cytotoxicity in renal epithelial cell internalization studies). A nonpolar mutation was introduced into the *flaD* gene, which encodes the flagellar cap protein. This mutation does not affect the synthesis of flagellin but rather prevents the assembly of an intact flagellar filament. In in vitro assays, the genetically characterized nonmotile mutant was found to be internalized by cultured human renal proximal tubular epithelial cells in numbers less than 1% of those of the flagellated parent strain. Internalization of the nonmotile mutant was increased significantly (14- to 21-fold) by centrifugation onto the monolayer. To assess virulence in vivo, CBA mice were challenged transurethrally with 10^7 CFU of *P. mirabilis* BA6163 (wild type) ($n = 16$), WPM111 (*hpmA* mutant) ($n = 46$), or BB2401 (*hpmA flaD* mutant) ($n = 46$). Differences in quantitative cultures between the parent strain and the hemolysin-negative mutant were not significant. However, the *hpmA flaD* mutant was recovered in numbers approximately 100-fold lower than those of the *hmpA* mutant or the wild-type parent strain and thus was clearly attenuated. We conclude that while hemolysin does not significantly influence virulence, flagella contribute significantly to the ability of *P. mirabilis* to colonize the urinary tract and cause acute pyelonephritis in an experimental model of ascending urinary tract infection.

Proteus mirabilis, an uncommon cause of urinary tract infection (UTI) in the normal host, is nevertheless recognized as a frequent cause of UTI in individuals with structural abnormalities of the urinary tract or chronic instrumentation, including catheterization (29, 30). Our group has been interested in developing a model for the pathogenesis caused by this bacterial species in the urinary tract. Putative virulence factors, including urease (9, 15, 18, 19, 20, 35, 44), mannose-resistant forward/*Proteus*-like (MR/P) fimbriae (4, 31), *P. mirabilis* fimbriae (also known as PMF) (25, 26), hemolysin (32, 36, 37, 38, 45, 46, 51), and amino acid deaminase (27), have been identified, and their contributions to virulence have been assessed by using in vitro and in vivo experimental models of infection.

P. mirabilis, once in the urinary tract, infects the kidney more commonly than does *Escherichia coli* (13). Acute pyelonephritis can be a consequence of this, and these infections of the kidney are often complicated by the formation of urease-induced renal stones (39) and bacteremia (41), suggesting that *P. mirabilis* is an invasive organism. That is, *P. mirabilis* can move from the urinary tract lumen to the bloodstream. Two factors could theoretically contribute to this facet of pathogenesis. Flagella could enable bacteria that have colonized the bladder to ascend the ureters to the renal tubules. Once the renal tubules are reached, HpmA hemolysin could lyse the tubular cells and allow bacteria access to the bloodstream.

We previously hypothesized that the severity of the histopathology was also associated with the internalization of *P. mirabilis* by human renal proximal tubular epithelial cells (HRPTEC). In the course of these studies, we noted that culture conditions that favored flagellation of *P. mirabilis* also favored internalization. It followed that flagella might play a role in the internalization by renal epithelial cells and, as a consequence, act to potentiate the virulence of the bacterium during ascending infection. We tested that hypothesis by constructing an isogenic mutant that is incapable of assembling an intact flagellum and used that mutant to measure internalization by HRPTEC as well as the ability to infect the bladder and kidneys of transurethrally challenged mice. Because of the inherent cytotoxicity of the HpmA hemolysin for HRPTEC, the flagellum-negative mutant was constructed in a hemolysin-negative background. This allowed us to measure the contribution of flagella to internalization in the absence of hemolysin-induced lysis of renal cells in the monolayer. We conclude from our studies that flagella contribute significantly to the ability of *P. mirabilis* to be internalized by cultured renal epithelial cells, colonize the urinary tract, and cause acute pyelonephritis in an experimental model of ascending UTI. In vivo, the contribution of hemolysin, as measured by numbers of infecting bacteria, was more subtle and was not found to be significantly different from that of the hemolysin-positive parent strain.

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TABLE 1. *P. mirabilis* strains used in this study

Strain	Phenotype or genotype	Reference(s)
BA6163	Wild-type isolate from catheter-associated bacteriuria; hemolytic, flagellated	32, 52
WPM111	HpmA hemolysin-negative mutant of BA6163	46
BB2000	Wild-type isolate; source of cloned *flaD*	8
BB2400	Rif^r isolate of strain WPM111	This study
BB2401	*flaD*-negative mutant of strain BB2400	This study

MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* strains and their isogenic mutants selected for internalization and virulence studies are listed in Table 1. Strain BA6163 was isolated from the urine of an elderly (≥65-year-old) woman with urinary-catheter-associated bacteriuria (32, 52). Strain WPM111, an isogenic HpmA hemolysin-deficient mutant of strain BA6163 (46), was used to study the involvement of hemolysin. The construction of strain BB2401, the flagellum-negative mutant of strain WPM111, is described in this report. *Salmonella typhimurium* ATCC 14028 and *E. coli* HB101 were used as positive and negative controls, respectively, in the gentamicin protection assay (11, 43) which was used to measure internalized bacteria.

Construction of a *P. mirabilis* mutant defective in flagellar filament synthesis by allelic-exchange mutagenesis. The strategy used to construct a flagellum-negative mutant of *P. mirabilis* is shown in Fig. 1. *P. mirabilis* WPM111 (an HpmA hemolysin-deficient mutant), which was chosen as the parental strain, is wild type for both swimming (Swm$^+$) and swarming motility and behavior (Swr$^+$) and is virulent in the CBA mouse model of ascending UTI (46). For purposes of counterselection, a spontaneous rifampin-resistant (Rif^r) mutant of WPM111 was obtained by spreading an overnight culture on nonswarming agar (consisting of [per liter] 10 g of tryptone, 5 g of yeast extract, 0.4 g of NaCl, 5 ml of glycerol, and 20 g of Bacto Agar) containing rifampin (100 μg/ml) and incubating for 24 h at 37°C. Among several Swm$^+$ Swr$^+$ Rif^r colonies, one isolate, designated BB2400, was selected for construction of the mutant.

P. mirabilis BB2400 was made defective in flagellar filament synthesis by using allelic exchange to incorporate a heterologous allele of *flaD*, an essential gene for flagellar filament synthesis (Fig. 1) (10, 17). The FlaD protein is necessary for the final polymerization of the flagellin monomer FlaA at the distal growing tip of the filament (5, 8). Loss of FlaD activity prevents the synthesis of the flagellar filament, although FlaA is actively produced and secreted into the medium. The *flaD* gene from *P. mirabilis* BB2000 has been previously described (8), and plasmid pDFFL1, in which the chloramphenicol resistance gene (*cam*) from Tn*5*-CM (12) was inserted into the cloned *flaD* gene, was constructed (5). Briefly, a FlaD mutant was constructed by subcloning a 1.2-kb *Hin*dIII fragment con-

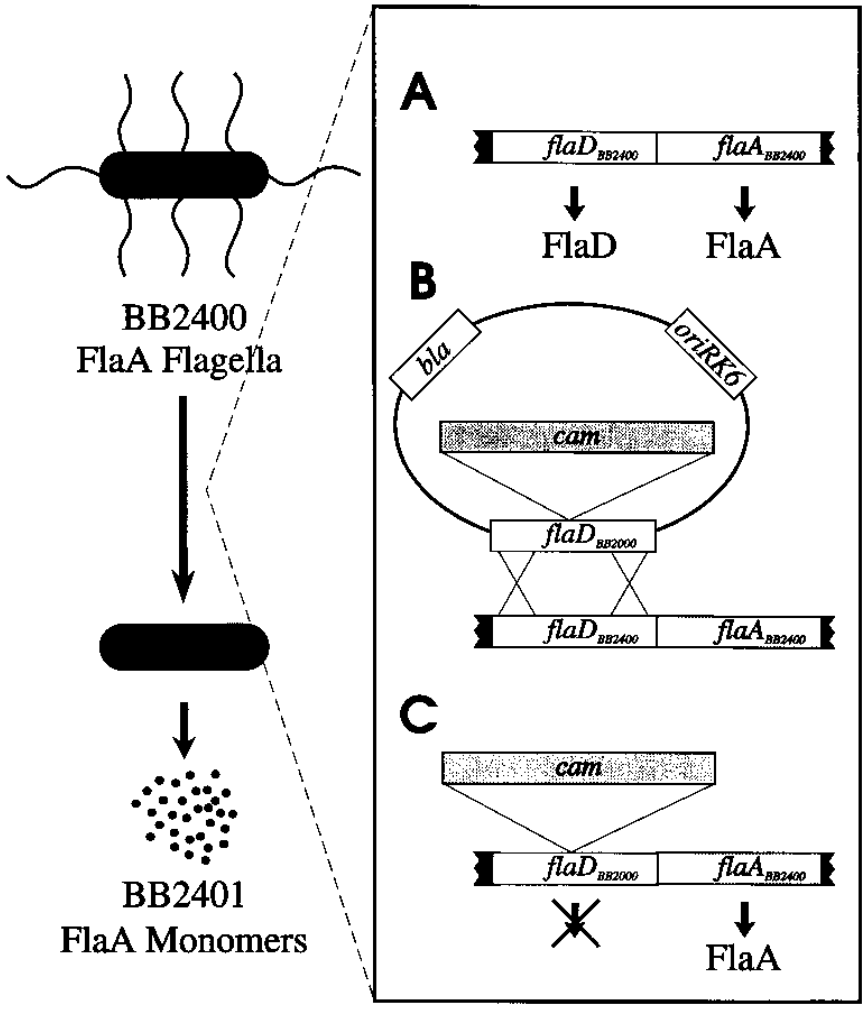

FIG. 1. Construction of a flagellar-filament-defective (Fla$^-$) *P. mirabilis* mutant. (A) *P. mirabilis* BB2400 (*hpmA* Rif^r) was mated with *E. coli* S17-1 (λ*pir*) harboring pDFFL1 (*flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$). An *ori*RK6 origin of replication (21) controls the replication of pDFFL1 such that pDFFL1 fails to replicate in strain BB2400 because of the absence of the *pir* gene product, and the (*flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$) construct either is restricted and destroyed or undergoes homologous recombination with *flaD*$_{BB2400}$. (B) Homologous recombination at the *flaD*$_{BB2400}$ locus results in allelic exchange between the chromosomal *flaD*$_{BB2400}$ and the plasmid-borne *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$. (C) The replacement of *flaD*$_{BB2400}$ with *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$ results in loss of FlaD activity and the failure of flagellin (FlaA) monomers to assemble at the distal tip of the flagellar filament. As diagrammed at the left, the resulting strain, BB2401, is Fla$^-$ because of the defective FlaD but still synthesizes FlaA.

taining the 5′ end of *flaD* and part of *flaA* and then blunt-end ligating this fragment into the *Eco*RV site in pGP704 (28). The ligation mixture was used to transform competent *E. coli* S17-1 (λ*pir*) with selection for the ampicillin resistance (Ap^r) conferred by pGP704. A *cam* gene was obtained from pUT/mini-Tn*5*-CM by *Hin*dIII digestion and electroelution of the *cam*-containing 3.4-kb fragment from an agarose gel. After mung bean nuclease treatment, the blunt-end *cam* gene fragment was ligated into the *Eco*RV site (nucleotide 270) within *flaD* (8) and the ligation mixture was used to transform S17-1 (λ*pir*), with selection for both Ap^r and chloramphenicol resistance (Cm^r). The construction was confirmed through restriction site mapping of the recombinant plasmid DNA, and a positive clone, referred to as pDFFL1, was chosen for further study.

The *flaD* suicide plasmid pDFFL1 was conjugally transferred from S17-1 (λ*pir*) to BB2400 by filter mating (6). *P. mirabilis* exconjugants were selected by plating on nonswarming agar containing chloramphenicol (40 μg/ml). Cm^r colonies were then screened for ampicillin sensitivity (Ap^s; i.e., loss of the plasmid), and a single ampicillin-susceptible colony was chosen. Genomic DNA from the gene replacement strain and from BB2400 was prepared by the spooling technique (42). Chromosomal DNA was digested with *Pvu*II, which cleaves asymmetrically in the *cam* gene, thereby providing a diagnostic test for replacement of the wild-type copy of *flaD* by the mutant construction. Southern blots of *Pvu*II-digested DNA hybridized to radiolabeled probes specific to the *cam* gene and $flaD_{BB2000}$ were used to confirm the mutagenesis. The $FlaD^-$ Fla^- strain BB2401 (containing $flaD_{BB2000}'$::*cam*::$'flaD_{BB2000}$) was then assayed for swimming motility, swarming motility and behavior, and the absence of flagellar filaments; the presence of flagellin monomers was assessed as described below.

Motility and differentiation analyses. The analysis of swarming motility and behavior was performed as previously described (7). In all swarming assays, Luria agar (containing [per liter] 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of Bacto Agar) plates were used after thorough drying at 42°C (45 to 60 min) to provide uniform conditions for evaluating swarming motility. A colony of the cells to be tested was incubated at 37°C for 14 to 16 h in 2 ml of Luria broth (which has the same formula as Luria agar but lacks agar) with the appropriate antibiotics added to the medium as required prior to inoculation. A 5-μl aliquot of an overnight culture was applied to the center of a dry Luria agar plate, and the inoculum was given sufficient time (usually less than 2 min) to be absorbed into the agar. Bacteria were incubated on the inverted plate at 37°C and examined at 4, 8, and 16 h postinoculation for swarming patterns. Strain BB2400 (which exhibits wild-type swarming) served as a control. Swarmer cell differentiation, i.e., the overproduction of flagella, cellular elongation, and polyploidy, was also examined microscopically as described by Belas et al. (7).

Swimming motility was assessed by both examination of the motility of cells grown in Mot broth (containing [per liter] 10 g of tryptone and 5 g of NaCl) by light microscopy and Mot agar (Mot broth plus 3.75 g of Bacto Agar per liter) analysis of chemotaxis. Media containing 0.375% agar are insufficiently viscous to induce swarmer cell differentiation yet allow undifferentiated swimmer cells to migrate through the agar gel matrix (7). A loopful of an overnight inoculum was gently stabbed into the center of a Mot agar plate. The plate was placed at 37°C, and swimming, i.e., outward movement of the cells through the agar matrix, was recorded at 4, 8, and 16 h after inoculation. The results of this test were confirmed by light microscopy of individual cells grown in Mot broth for 4 to 6 h at 37°C to a cell density of 10^7 to 10^8 cells per ml. BB2400 (which exhibits wild-type swimming) served as a control.

Electron microscopy. One drop of bacterial culture was placed directly on a Formvar-coated grid and negatively stained with 1% sodium phosphotungstic acid (pH 6.8). The specimens were examined in a JEOL 100B transmission electron microscope at an accelerating voltage of 80 kV.

Isolation of flagella and preparation of flagellin. Flagella from strain BB2400 were isolated by mechanical shearing, and the flagellin was purified from filaments by the procedures described by Belas et al. (7). Flagellin monomers secreted into the growth medium (L broth plus chloramphenicol) were obtained by taking 100 ml of cell-free supernatant from an overnight culture of BB2401 and concentrating the protein with Centriprep-30 and Centricon-30 concentrators (Amicon, Beverly, Mass.) in accordance with the manufacturer's recommendations.

Antisera to flagella. Antisera directed against flagella purified from *P. mirabilis* HU2450 and BB2000 were raised in New Zealand White rabbits as described previously (5).

Protein analyses and immunoblotting. Protein concentration was determined by using the bicinchoninic acid protein assay reagent as recommended by the manufacturer (Pierce Chemical Co.). Polyacrylamide gel electrophoresis (PAGE) was done as established by Laemmli (22). Western immunoblot and dot blot analyses of PAGE-denatured flagellar filaments from strain BB2400 and concentrated flagellin monomers from strain BB2401 supernatant were performed with rabbit anti-FlaC polyvalent antiserum as described previously (5, 7).

HRPTEC. Proximal tubular epithelial cells were isolated from kidney tissue obtained by autopsy (postmortem time, <24 h) at the University of Maryland School of Medicine and the medical examiner's office. HRPTEC were isolated, cultured, and characterized as described previously (50). Briefly, a kidney obtained at autopsy was perfused first with ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) to chelate calcium ions and weaken intercellular junctions and then with collagenase to dissolve the intercellular matrix. Outer cortical tissue was removed, minced, and further digested with a fresh collagenase solution. The resultant cell suspension was filtered through 40-μm-pore-size nylon mesh to remove glomeruli, and the volume was adjusted to a density of 10^6 cells per ml of cell medium. Cell medium consists of a balanced salt solution (Eagle's minimum essential medium + L-glutamine; Mediatech, Herndon, Va.) supplemented with the following constituents, all obtained from GIBCO (Grand Island, N.Y.): insulin (2 μM), heat-inactivated fetal bovine serum (10%), amphotericin B (1.25 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) (10 mM). The primary isolates were then plated and incubated at 37°C in an incubator with an atmosphere of 95% air–5% CO_2. The medium was changed twice a week. Once confluent, the cells were trypsinized, concentrated in cell medium containing 7% dimethyl sulfoxide, and stored in liquid nitrogen for subsequent use in experiments. Microtiter wells were seeded at a concentration of approximately 4×10^5 viable cells per ml of cell medium and grown to confluent monolayers in 48 to 72 h. HRPTEC were passaged no more than three times. HRPTEC cultures were examined by phase-contrast microscopy and stained for cytokeratins to verify the presence of epithelial cells.

Internalization assay. A commonly used gentamicin assay (43) was used to recover and enumerate intracellular bacteria internalized by cultured HRPTEC. Briefly, all *Proteus* strains and controls, grown in various media, were standardized to an optical density at 600 nm of 0.1 (1-cm light path; approximately 10^8 CFU/ml) in phosphate-buffered saline (PBS), pH 7.2, and 10 μl of the suspension (10^6 CFU) was overlaid onto washed HRPTEC monolayers in 100 μl of Eagle's minimal essential medium containing 1% glutamine (Bethesda Research Laboratories) but no other antibiotics or supplements. Monolayers either were or were not centrifuged (1,400 rpm, Sorvall H-1000B rotor, 3 min) and were then incubated at 37°C in a sealed container with a microaerobic atmosphere generated by a Campy Pouch (Becton Dickinson) for 3 h, washed three times with PBS, treated with gentamicin (100 μg/ml) for 1 h at 37°C, and washed again. Intracellular bacteria were released by incubation of the monolayer with a 1% Triton X-100 solution. Tenfold dilutions of the lysates were plated on Luria agar (11), and the plates were incubated for 18 h at 37°C. The results are expressed as CFU recovered per milliliter. A duplicate plate of the invasion assay was fixed with 70% ethanol after the last wash and stained with crystal violet to assess the integrity of monolayers.

CBA mouse model of ascending UTI. A modification (18) of the procedure of Hagberg et al. (16) was used to assess the virulence of parent and mutant strains of *P. mirabilis* HI4320. Six- to 8-week-old female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were used. Prior to challenge, spontaneously voided urine was collected in a sterile petri dish; bacteriuric mice were not used. Mice were challenged while anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.) by inserting a polyethylene catheter (2.5 cm long; outer diameter, 0.61 mm; Clay Adams, Parsippany, N.J.) into the bladder through the urethra and infusing 0.05 ml of a suspension containing 2×10^8 CFU/ml into the bladder over a 30-s period. The urethral catheter was removed immediately after challenge, and mice were cared for by the normal routine. Mice were inspected daily to monitor morbidity and mortality. Quantitative cultures of the urine, bladder, and kidneys were performed as previously described (18).

RESULTS

Immobilization of flagella and internalization by renal epithelial cells. Previous studies (1, 11) implicated motility in the process of internalization of bacteria by epithelial cells. To determine the effect of flagellum immobilization on internalization, strain WPM111 (HpmA hemolysin negative), isolated from the edge of a swarming culture on Luria agar, was or was not preincubated for 30 min with antiserum that had been raised in rabbits against purified flagella. Bacterial suspensions were overlaid onto monolayers of HRPTEC, and the numbers of internalized bacteria were estimated by the gentamicin assay after 0.5, 1, and 3 h (Fig. 2). At all antibody dilutions tested (1:1, 1:10, and 1:100), internalization of bacterial cells was dramatically inhibited by pretreatment of bacteria with antiserum.

Construction of a *P. mirabilis* Fla^- mutant defective in flagellar filament synthesis. To assess the contribution of flagella to virulence, a Fla^- mutant was constructed according to the scheme shown in Fig. 1. This strategy takes advantage of a previously cloned copy of the *P. mirabilis flaD* gene isolated from a closely related strain, BB2000 (5, 7, 8). FlaD is a capping protein for flagellar filament synthesis which functions to permit the polymerization of the monomeric flagellin protein as it travels through the hollow core of the flagellum to the distal end of the filament, where it is assembled (10, 17, 24).

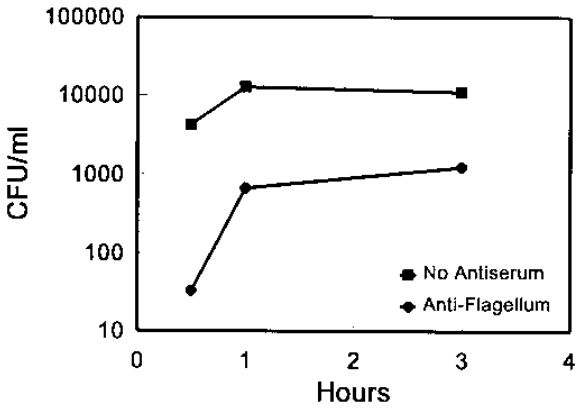

FIG. 2. Effect of flagellar immobilization on internalization of *P. mirabilis* by HRPTEC. *P. mirabilis* WPM111, isolated from the edge of a swarming culture on Luria agar, was or was not preincubated for 30 min with rabbit antiserum to *P. mirabilis* flagella (undiluted and at 1:10 and 1:100 dilutions). Suspensions were added to HRPTEC monolayers, and bacteria surviving treatment with gentamicin were quantitated. Data shown are those for the 1:10 dilution. Similar values were found for the 1:100 dilution.

FlaD$^-$ mutants therefore are unable to synthesize a filament and are phenotypically Fla$^-$ (and nonmotile), although they still synthesize wild-type levels of FlaA, which is secreted into the medium.

A 1.2-kb DNA fragment containing a portion of the *flaD* gene from BB2000 (*flaD*$_{BB2000}$) has been previously cloned, and its nucleotide sequence was determined (5, 8). Because of the extensive DNA-DNA homology between *flaD*$_{BB2000}$ and *flaD* of BB2400 (*flaD*$_{BB2400}$), we used *flaD*$_{BB2000}$ to mutate its homolog in BB2400. The mutagenesis employed an allelic exchange whereby a copy of *flaD*$_{BB2000}$ with a selectable chloramphenicol resistance marker (the Tn*5*-CM *cam* gene) inserted within the *flaD*$_{BB2000}$ coding region was cloned into pGP704 and then introduced by conjugal mating into BB2400. Following conjugal mating and transfer of the *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$ construct on pDFFL1 into strain BB2400, failure of plasmid replication resulted in loss of the plasmid. This loss was determined by screening Cm^r exconjugants for the Ap^s phenotype.

Confirmation of *flaD* double-crossover mutation in BB2400 was done by Southern blot hybridization of *Pvu*II-digested chromosomal DNA isolated from Cm^r Ap^s BB2400 exconjugants. Since *flaD*$_{BB2000}$ does not have a site for *Pvu*II cleavage and *cam* does (6, 8, 12), the replacement of *flaD*$_{BB2400}$ by *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$ was easily verified. Twelve Cm^r Ap^s BB2400 exconjugants were selected and screened for insertion of the *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$ construct. Eight of the 12 isolates were determined to have undergone allelic exchange and contained the *flaD*$_{BB2000}$′::*cam*::′*flaD*$_{BB2000}$ construct integrated into their chromosome in place of *flaD*$_{BB2400}$ (data not shown). One of these exconjugants, designated BB2401, was chosen for use in these studies.

Phenotypic analysis of the mutant. *P. mirabilis* BB2401 was analyzed for swimming and swarming motility, production of FlaA, and synthesis of a flagellar filament by comparison with the parent, BB2400. BB2401 was nonmotile, lacking both swimming and swarming motility, and failed to produce flagellar filaments when grown in broth (aerated or static) or on agar, as demonstrated by transmission electron microscopy (Fig. 3D to F). The presence of FlaA in cell-free supernatants from cultures of strains BB2400 and BB2401 was confirmed with FlaA-specific polyvalent antiserum (number 497 [5, 7]). BB2400 showed a strong positive signal in immunoblot dot blots of the concentrated supernatant, indicative of flagellar filaments normally shed during growth (7). BB2401 also showed a positive signal to FlaA-specific antiserum; however, much less FlaA was found in supernatants of BB2401 than in those of BB2400 (data not shown). This is most likely the result of proteolytic degradation of FlaA monomers, which are more susceptible to protease cleavage than are flagellar filaments, in which flagellin protein is protected from proteolysis in its polymeric form (8, 48, 49). These results confirm that BB2401 is defective in FlaD production and, as a consequence, is defective in synthesis of an intact flagellar filament (Fla$^-$).

Internalization of a flagellum-negative mutant. We compared the abilities of strains WPM111 and its isogenic nonflagellated mutant, BB2401, to be internalized by cultured HRPTEC (Table 2). Bacteria were cultured under conditions in which wild-type *P. mirabilis* strains would be both flagellated and fimbriated (i.e., aerated broth) (Fig. 3A), highly flagellated but not fimbriated (i.e., cells taken from the edge of a swarm on an agar plate) (Fig. 3B), or fimbriated only (i.e., passage in static broth) (Fig. 3C). Flagellated cells of WPM111 were internalized in large numbers (means of 22,420 and 26,000 CFU/ml), comparable to those of positive-control *S. typhimurium*. Nonflagellated WPM111 bacteria (static broth) yielded only about 1/10 as many internalized bacteria. The nonflagellated mutant, BB2401, was very poorly internalized under all culture conditions, resulting in values that were less than 1% of those of comparably cultured WPM111 ($P \leq 0.038$).

We postulated that poor internalization may simply be a function of the inability of the nonflagellated BB2401 to swim to the surface of the cultured epithelial cells. We reasoned that centrifugation of these bacteria onto a monolayer of epithelial cells might partially overcome this deficit by increasing the number of bacteria in contact with cells and thus augmenting internalization. Indeed, when BB2401 bacteria were centrifuged onto the renal epithelial cell monolayer, numbers of internalized bacteria were increased by 14- to 21-fold over the uncentrifuged control but were still much smaller than those of the flagellated strain WPM111 grown either in aerated broth ($P = 0.02$) or on agar ($P = 0.038$). By comparison, no significant increase (1.0- to 1.4-fold) in the number of internalized bacteria was afforded the flagellated WPM111 bacteria by centrifugation onto the monolayer prior to incubation and gentamicin treatment. These data implicated flagella as being necessary for significant levels of internalization but also suggested that the deficit amounted to more than just the loss of motility.

Contribution of flagella and hemolysin to virulence in experimental UTI. To evaluate the contribution of flagella and HpmA hemolysin to virulence in the urinary tract, we infected the bladders of CBA mice with 10^7 CFU of the parent (wild-type) strain BA6163, the *hpmA* mutant strain WPM111, or the *hpmA flaD* mutant strain BB2401 by transurethral challenge. At 1 week, the animals were sacrificed and urine, bladder, and kidneys were quantitatively cultured (Fig. 4). The *hpmA* mutation did not significantly affect the ability of the hemolysin-negative mutant to thrive in the urinary tract of the mouse, as determined by comparison with the values obtained for the parent strain (Fig. 4; Table 3). WPM111 bacteria were recovered from kidneys at concentrations similar to those of the wild-type strain BA6163. There were also no significant differences between the CFU per milliliter of urine or per gram of bladder tissue of the parent and the hemolysin-negative mutant.

On the other hand, the flagellum-negative, hemolysin-negative mutant BB2401 demonstrated a significant loss of virulence (ability to infect the bladder and kidney) when compared

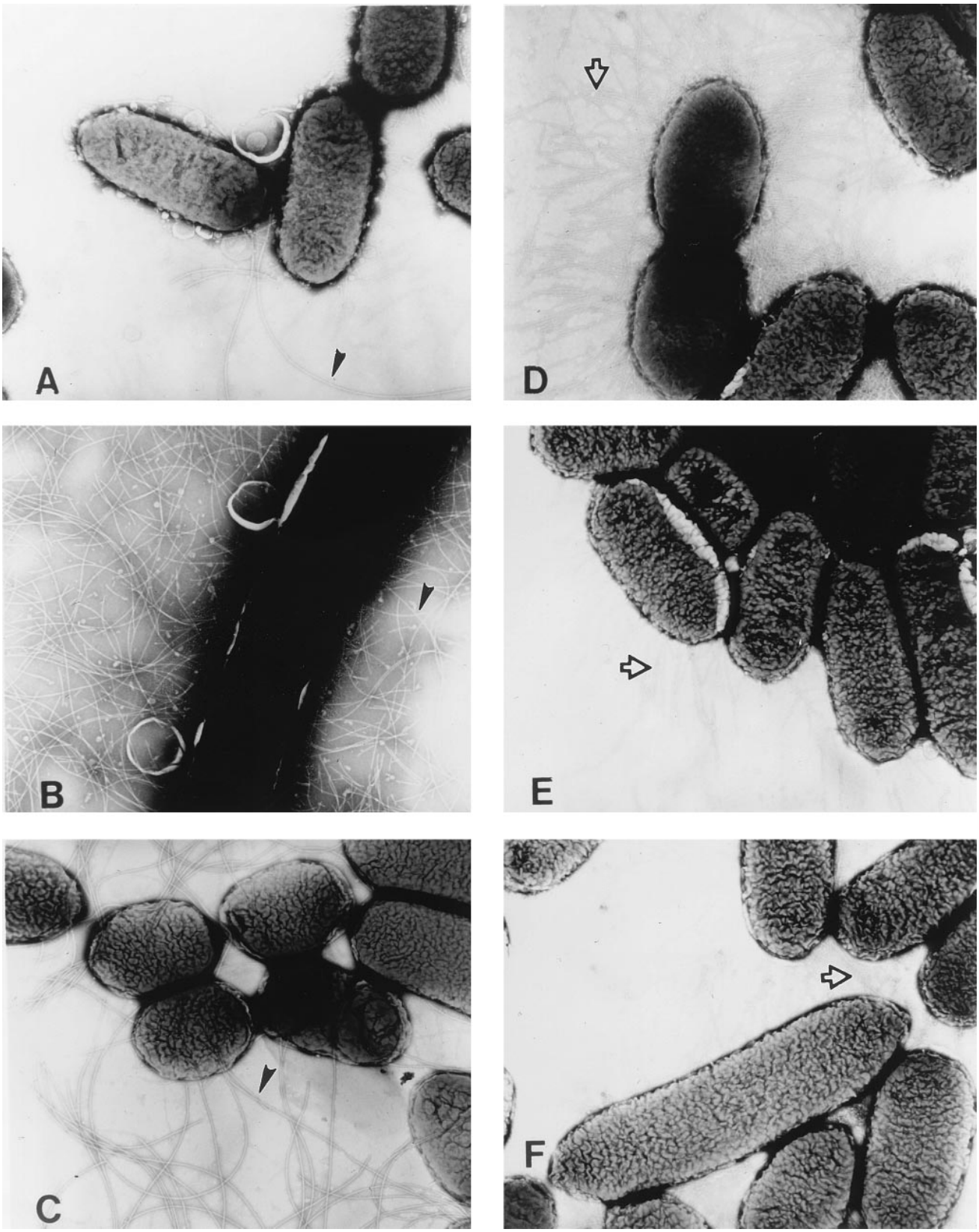

FIG. 3. Transmission electron microscopy of *P. mirabilis* and its flagellum-negative mutant. *P. mirabilis* WPM111 and BB2401 were grown under three sets of culture conditions (Table 2) and examined directly by transmission electron microscopy to confirm the phenotype with respect to fimbriation and flagellation. (A) WPM111 grown in aerated broth. (B) WPM111 taken from the edge of the swarm on Trypticase soy agar. (C) WPM111 grown statically in broth. (D) BB2401 grown in aerated broth. (E) BB2401 taken from the edge of the colony on Trypticase soy agar. (F) BB2401 grown statically in broth. Examples of flagella (closed arrowheads) and fimbriae (open arrows) are identified. Magnifications, ×18,600.

TABLE 2. Internalization of *P. mirabilis* strain by cultured human renal epithelial cells

Strain	Growth conditions	Internalized bacteria (CFU/ml)[a]		Fold increase after centrifugation (significance)[b]
		Without centrifugation	With centrifugation	
P. mirabilis WPM111	Broth; aerated; 18 h	22,420	32,020	1.4 (NS)
	Agar; swarm edge; 18 h	26,000	26,670	1.0 (NS)
	Broth; static; three passages, 48 h each	2,080	2,820	1.4 (NS)
P. mirabilis BB2401	Broth; aerated; 18 h	131 ($P = 0.02$)[c]	2,760 ($P = 0.02$)[c]	21.1 ($P < 0.001$)
	Agar; colony edge; 18 h	200 ($P = 0.009$)[c]	2,790 ($P = 0.038$)[c]	14.0 (NS)
	Broth, static; three passages, 48 h each	66 (NS)[d]	1,030 (NS)[d]	15.6 ($P = 0.039$)
E. coli HB101	Broth; aerated; 18 h	67	274	4.1 (NS)
S. typhimurium	Broth; aerated; 18 h	18,130	25,300	1.4 (NS)

[a] Values are means of three independent determinations.
[b] NS, not significantly different from counts without centrifugation when compared by the approximate *t* test for unequal variances. A *P* value is given when the difference was significant.
[c] Significantly different from strain WPM111 under identical culture and centrifugation conditions.
[d] NS, not significantly different from strain WPM111 under identical culture and centrifugation conditions.

with either the hemolysin-negative strain WPM111 or the parent strain BA6163. Quantitative cultures of the double mutant yielded roughly 100-fold fewer bacteria in urine, bladder, and kidneys than either the parent strain or the *hmpA* mutant. The number of mice colonized with $>10^3$ bacteria in the urine, bladder, and each kidney was significantly less for the flagella-negative, hemolysin-negative double mutant than for the hemolysin-negative mutant or the wild-type parental strain (Table 4).

DISCUSSION

The production of flagella appears to be critical for virulence of *P. mirabilis* in the urinary tract. A mutation in the gene encoding the FlaD capping protein prevented assembly of normally synthesized FlaA flagellin monomers and resulted in a loss of motility and swarming differentiation. When this mutation was constructed in an HpmA hemolysin-negative background, the resultant strain was significantly attenuated in the ability to colonize and infect the urinary tracts of experimentally challenged CBA mice (Fig. 4; Tables 3 and 4).

The contribution of flagella to virulence was suggested when we noted that culture conditions that favored the production of flagella also favored internalization of *P. mirabilis* by monolayers of cultured renal epithelial cells. When *P. mirabilis* was cultured in aerated broth or on plates which allowed all cells to be flagellated, bacteria were internalized in large numbers (Table 2), whereas poor internalization was noted when *P. mirabilis* was cultured statically in broth (i.e., not shaken; conditions under which cells are poorly flagellated) or when flagella were immobilized with specific antiserum (Fig. 2). While a number of phenotypes could be affected by these different culture conditions, one obvious difference was motility. These observations are consistent with those of Allison and col-

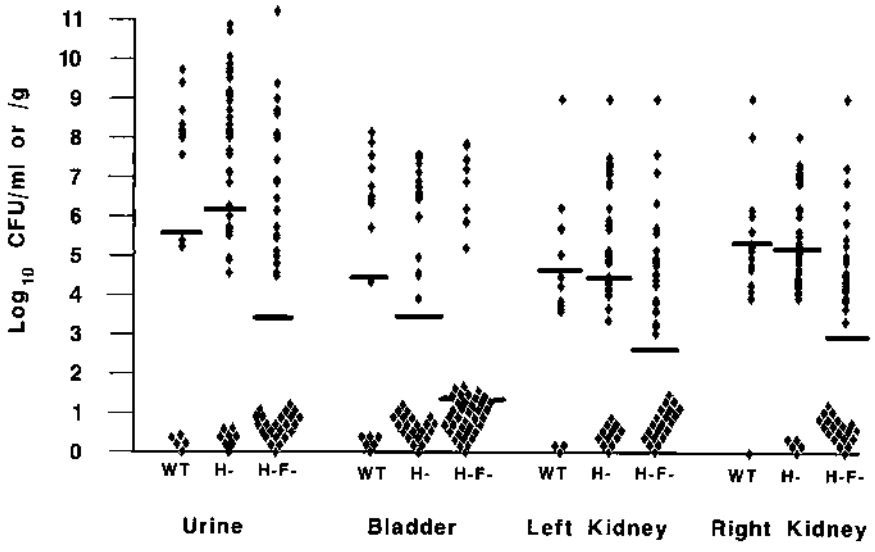

FIG. 4. Quantitative culture of *P. mirabilis* BA6163 and its hemolysin- and flagellum-negative mutants following transurethral challenge of CBA mice. Quantitative bacterial counts in urine and in bladder and kidney tissues were determined 1 week after mice were challenged with either *P. mirabilis* BA6163 (wild type) (WT), WPM111 (HpmA hemolysin-negative mutant of BA6163) (H−), or BB2401 (hemolysin-negative, FlaD flagellum-negative mutant) (H−F−). Each diamond represents the CFU per milliliter of urine or per gram of tissue from an individual mouse. The limit of detection was 10^2 CFU/ml of urine or g of tissue. Values along the *x* axis represent undetectable counts. Geometric means are shown as horizontal bars. *P* values are given in Table 3.

TABLE 3. Quantitative culture of *P. mirabilis* BA6163 and its *hpmA* and *flaD* mutants 1 week after transurethral challenge of CBA mice

Strain	No. of mice	Log_{10} CFU/ml of urine or g of tissue			
		Urine	Bladder	Kidney	
				Left	Right
BA6163 (wild type)	16	5.43	4.19	4.31	5.15
WPM111 (*hpmA* mutant)	46	6.22 (NS)[a]	3.44 (NS)	4.44 (NS)	4.97 (NS)
BB2401 (*hpmA flaD* mutant)	46	3.45[b] ($P = 0.0006$)	1.42[b] ($P = 0.0027$)	2.67[b] ($P = 0.0016$)	2.83[b] ($P = 0.0001$)

[a] NS, not significantly different from BA6163 (wild type).
[b] Significantly different from WPM111 (*hpmA* mutant).

leagues (1), who demonstrated diminished internalization by uroepithelial cells of transposon mutants with deficiencies in swarming motility.

To properly assess the role of flagella in internalization and virulence, we constructed by allelic exchange a mutant that was incapable of assembling flagella. Because of the presence of multiple flagellin genes (*flaA*, *flaB*, and *flaC*), we chose to disable flagellation by mutating the *flaD* gene, which would prevent the assembly of flagella (10) regardless of which flagellin gene was expressed. A chloramphenicol resistance gene was introduced into *flaD* in the chromosome of *P. mirabilis* WPM111 by allelic exchange using the suicide vector pGP704 (Fig. 1). Although this technique is not as straightforward as mutation of *E. coli* strains, this strategy has nevertheless been used for mutant construction in *P. mirabilis* (references 19 and 46 and this study). We have demonstrated that FlaA, which is directly downstream of the mutated *flaD*, is still synthesized in the mutant strain BB2401; hence, there are no apparent polar effects. Moreover, analysis of *flaD* and *flaA* from strain BB2000 indicates that these genes are transcribed from separate promoters and in opposite directions (5, 8). This organization is also found in *E. coli* and *S. typhimurium*, suggesting a highly conserved genetic structure and thus reinforcing our belief that the same organization exists in strain BA6163. That is, the synthesis of flagellin monomers, encoded by the gene directly downstream of the mutated *flaD*, is normal.

We observed that the flagellum-negative mutant BB2401 was very poorly internalized by HRPTEC (Table 2). We hypothesized that the ability of the bacteria to be internalized could be restored by bringing the bacteria into contact with the monolayer by centrifugation. Indeed, the number of internalized bacteria was increased 14- to 21-fold following centrifugation. Although these increases were dramatic compared with the 1- to 1.4-fold increases achieved for the flagellated strain WPM111 after centrifugation, the absolute numbers of the nonmotile strain never reached more than about 1/10 those of the motile strain. This suggested that loss of motility was not the only factor affected by the mutation and was not entirely responsible for poor internalization.

There is a great deal of evidence that functional flagella are necessary for sensing cues from the environment and that through interaction with their surrounding milieu they can trigger activation of other genes, including those encoding hemolysin (irrelevant here because of the use of the hemolysin-negative mutant), urease, protease, and probably additional, as yet unrecognized genes (3). Thus, the mutation in *flaD*, while not having direct polar effects, may nevertheless have pleiotropic effects on gene expression because of the lack of flagella, which sense medium viscosity and initiate a signal transduction pathway that results in expression of additional genes.

In the animal studies, the flagellar mutation clearly attenuated *P. mirabilis*, the effect being more pronounced in the bladder than in the kidney (Tables 3 and 4). Nevertheless, there were significant differences in the percentage of mice with $>10^3$ CFU/ml of urine or g of tissue between nonmotile BB2401 and motile WPM111 in the urine, bladder, and kidneys.

The link between flagella and virulence has been observed previously for *P. mirabilis*. Zunino and colleagues (54) found that a naturally occurring nonmotile strain was less virulent than motile strains in a mouse model of ascending UTI. While these represented different strains that were not isogenic, the authors were able to conclude that flagella were not absolutely required for virulence, an observation supported in our study by the successful infection of some mice by the flagellum-negative mutant. When examined in other bacterial species, the loss of the ability to produce flagella generally results in a less virulent organism, whether the flagellum-negative strains are spontaneous mutants, transposon mutants, phase variants, or specifically constructed isogenic mutants. Nonflagellated isolates of *S. typhimurium* (23), *Erwinia carotovora* (34), *Campylobacter jejuni* (14), *Bordetella avium* (33), *Bartonella bacilliformis* (40), *Bacillus thuringiensis* (53), and *Clostridium chauvoei* (47) were all found to be less invasive or less virulent than

TABLE 4. Infection caused by *P. mirabilis* BA6163 and its hemolysin- and flagellum-negative mutants following transurethral challenge of CBA mice

P. mirabilis strain	No. of mice/total (%) with $>10^3$ CFU/ml of urine or g of tissue[a]			
	Urine	Bladder	Kidney	
			Left	Right
BA6163 (wild type)	11/16 (69)	10/16 (63)	13/16 (81)	15/16 (94)
WPM111 (*hpmA* mutant)	37/46 (80)	24/46 (52)	37/46 (80)	41/46 (89)
BB2401 (*hpmA flaD* mutant)	24/46 (52) ($P = 0.008$)[b]	10/46 (22) ($P = 0.005$)[b]	27/46 (59) ($P = 0.04$)[b]	28/46 (61) ($P = 0.004$)[b]

[a] Determined 1 week after urethral challenge.
[b] Compared with strain WPM111 by chi-square analysis. *P* values of <0.05 are shown; otherwise *P* is >0.05.

the parental strains in their respective in vitro and in vivo models of pathogenesis.

In the present study, it must be emphasized that the flagellar mutation was constructed in a hemolysin-negative background. In a previous study (11), we found that in order to study the internalization of *P. mirabilis* by cultured HRPTEC, hemolysin could not be present. Hemolysin-positive strains, when overlaid onto HRPTEC, rapidly lysed cells within the monolayer, rendering the results of the gentamicin protection assay meaningless. Therefore, we found that the assessment of the contribution of flagella to internalization must be done in a hemolysin-negative background.

Clearly, double mutation of *hpmA* and *flaD* attenuates *P. mirabilis* (Fig. 4). While the mutation in hemolysin alone did not result in a statistically significant drop in quantitative cultures in the mouse model of ascending UTI, the additional mutation in flagellum assembly critically limited the ability of the organism to colonize and infect the urinary tract (Tables 3 and 4).

On the other hand, the mutation of *hpmA* did not significantly attenuate *P. mirabilis*. The finding that a mutation in hemolysin does not critically affect virulence in experimental infection is consistent with other studies. Swihart and Welch (46), who constructed the *hpmA* mutation in strain BA6163, demonstrated no significant difference between mutant and parent in quantitative culture or histologically assessed kidney damage of transurethrally challenged mice 1 week or less after challenge (data not shown). Likewise, Allison and coworkers (2) showed no difference in kidney involvement when comparing an uncharacterized nonhemolytic transposon mutant of *P. mirabilis* with its hemolytic parent strain, U6450. As in the previous study (46), we were unable to demonstrate a statistical difference in quantitative culture of urine and of bladder and kidney tissues between hemolysin-negative strain WPM111 and parent strain BA6163. There was not even a trend toward lower bacterial counts in animals infected with the mutant. This suggests that the effect of hemolysin in the kidney may be subtle and difficult to measure in the mouse model.

We conclude that the production of flagella is essential for *P. mirabilis* virulence in the urinary tract. The attenuation conferred by the mutation, however, may not be merely the result of the loss of motility but rather may be due also to the inability to properly regulate expression of other genes, some of which may be directly involved in virulence.

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