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Internalization of *Proteus mirabilis* by Human Renal Epithelial Cells

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***Proteus mirabilis*, a common agent of bacteriuria in humans, causes acute pyelonephritis and bacteremia. Renal epithelium provides a barrier between luminal organisms and the renal interstitium. We have hypothesized that *P. mirabilis* may be internalized into renal epithelium. To test this hypothesis, we added suspensions of three *P. mirabilis* strains (10^8 CFU) to confluent monolayers of primary cultures of human renal proximal tubular epithelial cells (HRPTEC) and, after 3 h, found the bacteria internalized within membrane-bound vacuoles by light and electron microscopy. Internalization of bacteria by HRPTEC was corroborated by using the gentamicin protection assay. Cytolysis of HRPTEC by the HpmA hemolysin, however, was a confounding factor in this assay, and therefore a hemolysin-negative *hpmA* mutant was used in subsequent experiments. The nonhemolytic mutant WPM111 did not disrupt the monolayer and was recovered in numbers that were 10- to 100-fold higher than those of the hemolytic parent BA6163. Cytochalasin D (20 μg/ml) inhibited internalization of *Salmonella typhimurium* but not that of *P. mirabilis*, suggesting that the latter species enters HRPTEC by a mechanism that is not dependent on actin polymerization. We suggest that HpmA hemolysin-mediated cytotoxicity and internalization of bacteria by HRPTEC may play a role in the development of *Proteus* pyelonephritis.**

Proteus mirabilis is a common cause of urinary tract infections in patients with structural abnormalities of the urinary tract (20). Even in the normal host, *P. mirabilis* is the second or third most common organism causing urinary tract infection and acute pyelonephritis (20). The organism has been found to localize preferentially in the kidney (8, 23), is frequently associated with stone formation (10), can cause severe damage to the kidney epithelium (5), and is an important agent of bacteremia (21). Most infections are ascending, and the bacterium must first gain access to the bladder, ascend the ureter to the renal pelvis, and there, or in the tubular collecting system, interact with epithelium to invade the kidney.

A step in this invasion or in the process of host defense may be internalization of the organism into renal epithelial cells. Evidence that *P. mirabilis* can enter and multiply intracellularly within kidney epithelial cells was first presented by Braude and Siemienski (5) in light micrographs of cell imprints of kidney tissue from experimentally infected rats. Other investigators have demonstrated that strains of *P. mirabilis* can invade transformed cell lines and have implicated certain bacterial factors in this process. Peerbooms et al. (17), using relatively nonspecific methods of chemical mutagenesis and general protein inhibition, suggested that the hemolysin of *Proteus* spp. plays a role in invasion of Vero cells. Additionally, Allison et al. (1, 2) observed that *P. mirabilis* swarm cells with increased motility, hemolysin, urease, and protease activity invaded a urothelial cell line in higher numbers than did normal vegetative cells of this strain.

The purpose of this study was twofold: (i) to use putative target cells of acute pyelonephritis, human renal epithelial cells, to confirm internalization of *P. mirabilis*; and (ii) to use an isogenic mutant to focus specifically on hemolysin and its role in internalization. Our results, using human cells, differ markedly from those of previous studies.

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MATERIALS AND METHODS

Bacterial strains. *P. mirabilis* strains and their isogenic mutants selected for internalization studies are listed in Table 1. Strains HI4320 and BA6163 were isolated from the urine of elderly (≥65 years old) female patients with Foley catheters in place for ≥30 days (27). Strain WPM111, an HpmA hemolysin-deficient derivative of strain BA6163 (24, 25), was used to study the involvement of hemolysin. Strain CFT322 was isolated from a patient admitted to the University of Maryland Hospital with clinical symptoms of acute pyelonephritis (12). *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* HB101 were used as positive and negative controls, respectively, in the gentamicin protection assay. *E. coli* CFT073 *hlyD*::Tn*phoA*, a hemolysin-negative mutant of a P-fimbriated pyelonephritogenic strain, was used as the positive control for adherence assays and has been described previously (14).

Media and growth conditions. Bacteria were grown aerobically by inoculation into Luria broth (11) with incubation at 37°C without shaking for 48 h. The third serial passage was used for internalization studies. For anaerobic growth, bacteria were inoculated into prereduced thioglycolate broth and incubated without shaking at 37°C in an anaerobe jar with H_2 Pak (BBL).

For comparison of bacteria cultivated in urine versus Luria broth, each strain was inoculated and serially passaged in Luria broth and filter-sterilized pooled morning urine containing 10% Luria broth. The cultures were incubated for 48 h at 37°C in an anaerobic jar with a microaerobic atmosphere generated by a CampyPak (Becton Dickinson).

To compare swarm cells with nonswarming vegetative cells, bacteria were isolated from the outer edge of an active swarm mass (20) grown on Trypticase soy agar (Becton Dickinson) at 37°C and used immediately for invasion assays. Nonswarming

TABLE 1. Bacterial strains used in this study

Bacterium	Strain	Relevant phenotype	Source	Reference(s)
P. mirabilis	Hl4320	Wild type	Urine (catheter)	12, 27
	BA6163	Wild type	Urine (catheter)	12, 24, 25, 27
	WPM111	Hemolysin negative	Mutant of BA6163	13, 25
	CFT322	Wild type	Urine (pyelonephritis)	14
S. typhimurium	ATCC 14028	Positive control for gentamicin assay		20
E. coli	CFT073 *hlyD*::Tn*phoA*	P fimbriated, hemolysin negative	Tn*phoA* mutant of strain CFT073	14
	HB101	Negative control for gentamicin assay	Laboratory strain	14

cells were isolated from bacteria grown overnight on nonswarming Luria agar containing 0.5% glycerol and 2% agar (4). Metabolically less active, nonswarming vegetative cells were isolated from nonswarming agar cultured overnight and held for 72 h at 4°C. Also, nonswarming cells from WPM111 were recovered from the center of a swarming colony.

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. Human renal proximal tubular epithelial cells (HRPTEC) were isolated, cultured, and characterized as described previously (26). Briefly, kidney obtained at autopsy was perfused first with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] 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 nylon mesh (40 μm) to remove glomeruli, and the volume was adjusted to a cell density of 10^6 cells per ml of Cellgro medium, consisting of Eagle's minimum essential medium plus L-glutamine (Mediatech, Herndon, Va.) supplemented with the following constituents obtained from GIBCO (Grand Island, N.Y.): insulin (1.0 U/ml), heat-inactivated fetal bovine serum (10%), amphotericin B (1.25 μg/ml), penicillin (1.0 U/ml), streptomycin (100 μg/ml), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (10 mM). The primary isolates were then plated and incubated at 37°C in an atmosphere of 95% air–5% CO_2. Medium was changed twice a week. Once confluent, the cells were trypsinized, concentrated in medium containing 7% dimethyl sulfoxide, and stored in liquid nitrogen for subsequent use in experiments. Microtiter wells or electron microscopy dishes were seeded at a concentration of approximately 4×10^5 viable cells per ml and grown to confluent monolayers for 48 to 72 h in Eagle's minimum essential medium containing 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). HRPTEC were passaged no more than three times. Before each experiment, HRPTEC cultures were examined by phase microscopy to ensure the cobblestone appearance of the monolayer.

Adherence assay. *P. mirabilis* strains were passaged three times for 48 h each in Luria broth without shaking at 37°C. Bacteria were harvested by centrifugation (10,000 × *g*, 10 min, 4°C) and suspended in CMSS (10 mM HEPES, 0.44 mM KH_2PO_4, 0.37 mM Na_2HPO_4, 137 mM NaCl, 5.37 mM KCl, 0.81 mM $MgSO_4$, 1.37 mM $CaCl_2$, 1% glucose; adjusted to pH 7.2) so that the optical density at 550 nm (1-cm path length) equalled 0.62 to 0.68. Bacterial suspension (300 μl) was added to chamber slides (eight well; Nunc) containing monolayers of HRPTEC. No antibiotics were included in the medium. Slides were incubated with gentle rocking at 37°C. After 15, 30, 60, 90, 120, 150, and 180 min, suspensions were aspirated and washed three times with 400 μl of CMSS. Monolayers were fixed with 70% ethanol and stained for 10 min with a 1:50 dilution of Giemsa stain. Slides were observed under oil immersion (×1,000).

Internalization assay. A commonly used gentamicin assay (22) was used to recover intracellular bacteria. Briefly, all *Proteus* strains and controls, grown in various media, were standardized to an optical density at 600 nm of 0.1 (1-cm path length; approximately 10^8 CFU) in phosphate-buffered saline (PBS; pH 7.2) and overlaid onto washed HRPTEC monolayers in Eagle's minimum essential medium containing 1% glutamine (Paragon) but no other antibiotics or supplements. Monolayers were incubated at 37°C in a Campy pouch (BBL), washed three times with PBS, treated with gentamicin (100 μg/ml), 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 incubated for 18 h at 37°C. Results are expressed as CFU recovered per milliliter. Inhibition studies using cytochalasin D (6) were done by preincubating HRPTEC with the reagent for 30 min prior to adding bacteria. 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.

Epithelial cell cytotoxicity was measured by a modification (7) of the neutral red assay described by Babich and Borenfreund (3).

Transmission electron microscopy. Invasion assays were done on HRPTEC grown to a confluent monolayer in Lux Permanox dishes (Thomas Scientific). At 3 h, monolayers were treated with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. After postfixation with 2% aqueous OsO_4, the monolayers were dehydrated through an ethanol series, cleared in propylene oxide, and embedded in Polybed-812. Ultrathin sections were mounted on carbon-coated grids, double stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

Hemolytic titer. Serial twofold dilutions of a 50-μl sample from the gentamicin protection assay were made with 0.85% NaCl in microtiter dishes. An equal volume of a 1% (vol/vol) suspension of sheep erythrocytes (Morris Farms, Cockeysville, Md.) was added to each well. Plates were incubated statically at 37°C and read macroscopically after 1 h. The hemolytic titer was defined as the reciprocal of the last dilution in which complete hemolysis was observed.

RESULTS

***P. mirabilis*-HRPTEC association.** A bacterial suspension (approximately 10^8 CFU) of *P. mirabilis* was added to confluent monolayers of HRPTEC. Monolayers, washed and stained at times ranging from 15 to 180 min after addition of bacteria, were observed by light microscopy. The positive control, a hemolysin-negative Tn*phoA* mutant of P-fimbriated *E. coli* CFT073 (14), associated in high numbers with the washed monolayers, attaching to virtually every cell. In contrast, *P. mirabilis* Hl4320 and BA6163 adhered in very low numbers to HRPTEC. However, after 60 min, adherence was very difficult to quantitate because very few HRPTEC remained in the monolayer, only at the border of the chamber.

We assume that the monolayer damage was due to hemolysin expressed in both of these strains, a phenomenon we have previously demonstrated (13). Therefore, we used an isogenic hemolysin-negative mutant of strain BA6163, namely, WPM111, to observe adherence over longer time periods. This hemolysin-negative mutant allowed HRPTEC to persist for 120 min. However, numbers of adhering bacteria were still low even at the later time points. These qualitative observations demonstrated that, compared with a pyelonephritogenic *E. coli* strain, relatively few *P. mirabilis* organisms associated with (i.e., adhered to or internalized into) human renal epithelial cells and that HpmA hemolysin, a cytotoxin produced by all wild-type isolates of *P. mirabilis* (13, 16, 18, 19, 24, 25), would represent a serious confounding factor in studies of the interaction with HRPTEC.

Internalization observed by electron microscopy. Light microscopy demonstrated some *P. mirabilis* bacteria that appeared to be in vacuoles of surviving HRPTEC. However, these findings were ambiguous and prompted us to examine the association of *P. mirabilis* in HRPTEC by electron microscopy. HRPTEC monolayers were inoculated with *P. mirabilis* Hl4320 serially passaged under static conditions for 48 h in Luria broth. Bacteria cultured under these conditions were hemolytic but did not have the high hemolytic titer associated with elongated swarm cells cultured on agar surfaces (2). Following incubation for 3 h, monolayers were washed and fixed. Transmission electron microscopy of thin sections of these HRPTEC monolayers confirmed the presence of internalized bacteria within membrane-bound vacuoles. In Fig. 1A, one bacterial cell is seen at some distance from the cell, one is seen in close proximity to the cell but not intimately attached, and one is seen within a membrane-bound vacuole inside the cell. In Fig. 1B, three membrane-bound vacuoles, contained within the cell, envelop *P. mirabilis* bacteria. In one vacuole, there is the appearance of a dividing bacterium; this kidney cell appeared to be severely compromised. To gain a sense of the frequency with which this internalization occurs, we examined all HRPTEC on one electron microscope grid. For multiple thin sections of 12 HRPTEC, we found one cell with four internalized bacteria.

In many of the experiments with this hemolytic strain, there was again evidence of monolayer destruction. In a specific example (Fig. 1C), an electron micrograph of a thin section of an embedded culture well that had contained both HRPTEC monolayers and *P. mirabilis* Hl4320 revealed only bacterial cells remaining after 3 h.

Cytotoxicity confounds internalization assay. To quantitate internalization of *P. mirabilis* into HRPTEC, we used a standard gentamicin protection assay (22) with *P. mirabilis* wild-type strains Hl4320, BA6163, and CFT322. *S. typhimurium* ATCC 14028 and *E. coli* HB101 were used as positive and negative controls, respectively. HRPTEC cultures were overlaid with bacterial suspensions (10^8 CFU) and incubated for 3 h prior to gentamicin addition (Fig. 2). As expected, *S. typhimurium* was recovered in high numbers, significantly more than all other strains ($P < 0.05$). *P. mirabilis* Hl4320, BA6163, and CFT322 were not internalized in significantly higher numbers than *E. coli* HB101 ($P \geq 0.14$). However, that this finding was spurious was evident by light microscopy, which revealed that only a fraction of the cells incubated with *P. mirabilis* strains remained in the monolayers while control strains, *S. typhimurium* and *E. coli*, did not show significant damage. Therefore, a small number of remaining HRPTEC accounted for all of the gentamicin-protected *P. mirabilis* bacteria, whereas much of the initial monolayer was still available to the control strains for protection from gentamicin.

To confirm this, in parallel with the standard gentamicin assay, culture plates containing HRPTEC were exposed to *P. mirabilis* strains for various times (15 to 180 min) and then stained with crystal violet. By light microscopy, retention of crystal violet dye correlated with the presence of a confluent monolayer. For *P. mirabilis* Hl4320, BA6163, and CFT322, culture wells stained a lighter blue or retained no dye whatsoever after 20 min. Control wells containing no bacterial inoculum stained a dark blue color easily visible without microscopy. *P. mirabilis* WPM111, the hemolysin-negative mutant of strain BA6163, did not cause monolayer destruction; these cells retained a consistent dark blue stain comparable to the control wells even after 3 h.

Internalization of hemolysin-negative mutant. Internalization of the hemolysin-negative mutant demonstrated dramatically why the cytotoxicity of HpmA hemolysin is a confounding factor in the measurement of internalization of *P. mirabilis* by kidney cells that are simultaneously being lysed. To avoid this effect, we used a hemolysin-negative mutant of strain BA6163, i.e., strain WPM111, to measure internalization by the gentamicin assay. The internalization of these and other bacteria by HRPTEC was measured over time from 1 to 4 h (Fig. 3). A 1-h incubation of bacteria followed by 1 h of gentamicin exposure resulted in *S. typhimurium* being recovered from HRPTEC in the highest numbers and *E. coli* HB101 being recovered in the lowest numbers (Fig. 3). Over time, *P. mirabilis* BA6163 was recovered in progressively lower numbers, and after 4 h these numbers were as low or lower than those obtained for *E. coli* HB101 (Fig. 3). This decrease in recovery of viable bacteria was presumed to be a result of HpmA-induced cytotoxicity. The hemolysin-negative strain WPM111, on the other hand, was recovered in progressively higher numbers over the course of the experiment. The difference of the means between WPM111 and BA6163 was significantly different at 3 ($P = 0.0014$) and 4 ($P = 0.031$) h.

To further measure the effect of HpmA hemolysin on internalization, strain BA6163 and its isogenic hemolysin-negative mutant WPM111 were cultured under a variety of conditions and used to challenge HRPTEC monolayers (Fig. 4). Bacteria were grown in urine or Luria broth, under aerobic or anaerobic conditions, and on agar on which bacteria were allowed to swarm or were inhibited from swarming. Consistently, the nonhemolytic mutant (WPM111) was recovered in numbers that were 10- to 100-fold higher than those of the hemolytic parent (BA6163) following 3 h of incubation with HRPTEC cultures. The largest difference was observed between the wild type and the mutant when the bacterial inoculum was prepared from the edge of an actively swarming culture. The hemolysin titer of BA6163 was highest (titer = 1:32) under these culture conditions (see Fig. 4 legend for other titers). The only exception to this relationship was observed when bacteria were cultured on nonswarming agar;

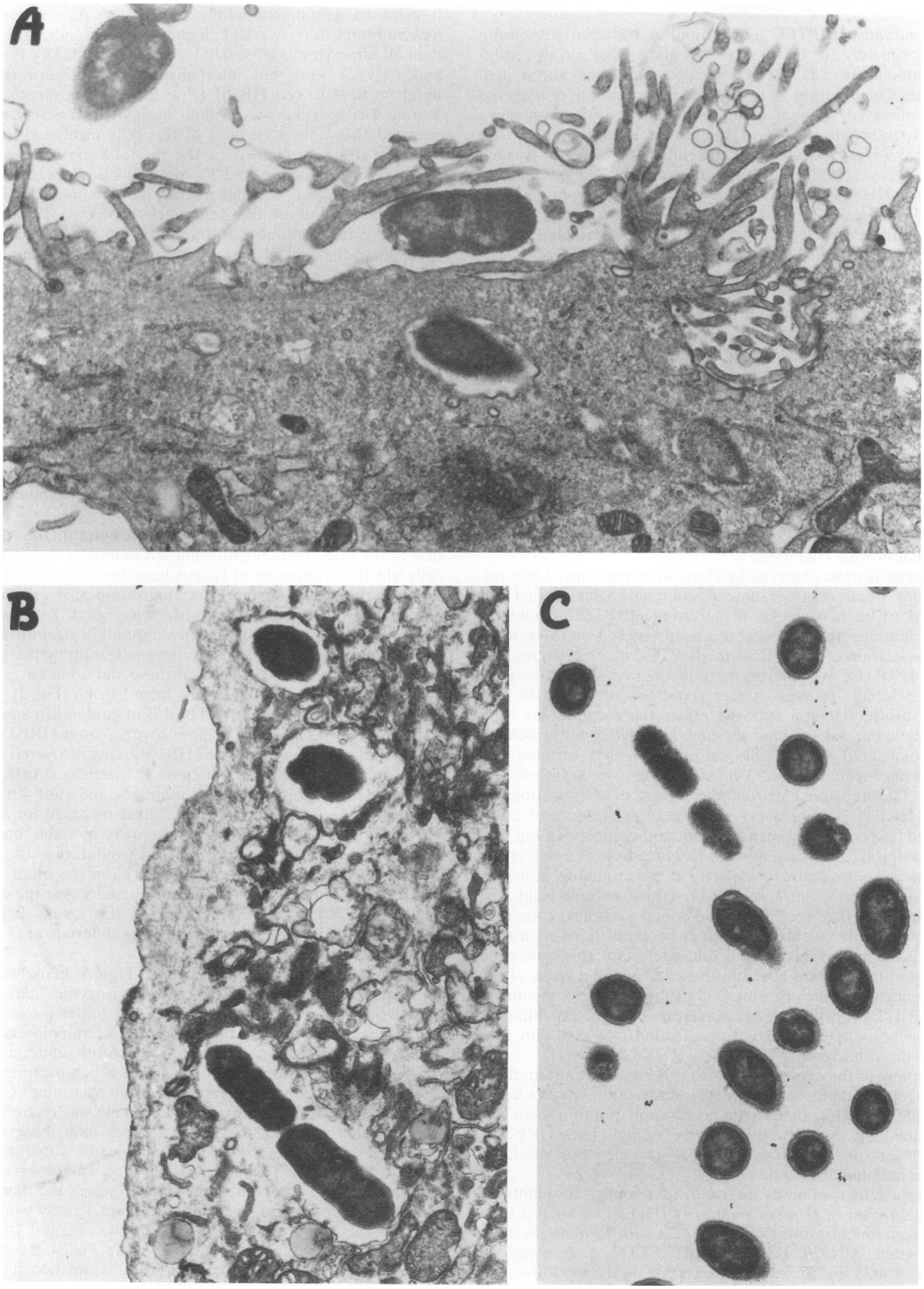
A
B
C

FIG. 1. Transmission electron microscopy of thin sections of HRPTEC following incubation with *P. mirabilis*. Suspensions of *P. mirabilis* (approximately 10^8 CFU) were overlaid onto HRPTEC monolayers and incubated at 37°C. After 3 h, monolayers were washed, fixed with glutaraldehyde, subjected to thin sectioning, and examined by transmission electron microscopy. (A) Strain grown statically in Luria broth; magnification, ×21,600. (B) Strain grown in urine; magnification, ×18,090. (C) Strain isolated from the edge of an actively swarming culture on Trypticase soy agar; magnification ×16,200.

the numbers of internalized bacteria were not significantly different for parent and mutant strains ($P > 0.05$).

Effect of cytochalasin D on internalization. Our data suggested that *P. mirabilis* internalization is a feature best examined when the confounding effect of hemolysin is removed. We hypothesize that internalization was effected by actin polymerization in a phagocytosis-like action by the HRPTEC. To test this hypothesis, HRPTEC monolayers were preincubated with cytochalasin D (0, 0.2, 2, 10, and 20 μg/ml) for 30 min prior to addition of bacteria. Internalization of *S. typhimurium* ATCC 14028 was found to be inhibited in a dose-dependent manner. A concentration of 20 μM yielded markedly low internalization of this species. At 3 h, only 10% of the number of *S. typhimurium* internalized into HRPTEC in the absence of cytochalasin D survived the gentamicin treatment. To demonstrate that the reduction in internalization of *S. typhimurium* was not due to the cytotoxic effect of cytochalasin D on HRPTEC, a neutral red retention assay was done in the presence of the same concentrations of cytochalasin D. After 3 h of incubation with the inhibitor, there were no significant differences ($P > 0.1$) in the optical densities (0.085, 0.090, 0.081, 0.089, and 0.092) of the neutral red dye retained by HRPTEC cultures exposed to 0, 0.2, 2, 10, and 20 μM, respectively, of inhibitor.

In subsequent experiments, 20 μM of cytochalasin D was used to measure the effect on internalization of *P. mirabilis* BA6163 and WPM111 and *S. typhimurium* 14028 compared with the number of bacteria internalized for these strains in the absence of the inhibitor (Table 2). In contrast to *S. typhimurium*, internalization of *P. mirabilis* BA6163 and its hemolysin-negative mutant was not significantly inhibited by cytochalasin D ($P > 0.1$).

To confirm these results, the internalization of *P. mirabilis* WPM111 was measured after 3 h of incubation with HRPTEC in two additional experiments, each in triplicate. At 0, 0.2, 2, 10, and 20 μM of cytochalasin D, 362, 457, 460, 262, and 372 CFU/ml were recovered after gentamicin treatment. These values were not significantly different, again demonstrating that cytochalasin D does not inhibit internalization of this *P. mirabilis* strain.

DISCUSSION

Strains of *P. mirabilis* are internalized by cultured human renal epithelial cells. In the host, these human cells represent a thin barrier that, in an ascending urinary tract infection, may prevent bacteria from entering the kidney wherein they may establish an acute pyelonephritis. Bacteria that enter these cells are found within membrane-bound vacuoles, as suggested

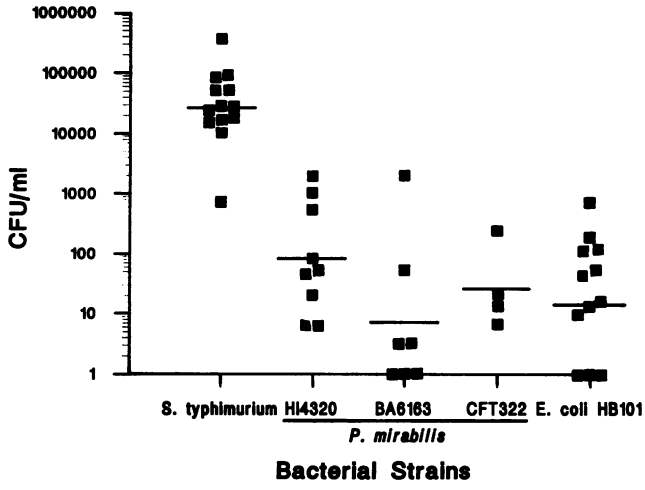

FIG. 2. Internalization of wild-type *P. mirabilis* strains by HRPTEC as measured by the gentamicin protection assay. Suspensions of bacterial strains (10^8 CFU), passaged statically in Luria broth, were overlaid onto monolayers of HRPTEC and incubated for 3 h at 37°C. Monolayers were treated with gentamicin (100 μg/ml), washed, and lysed with Triton X-100. The number of CFU of lysate per milliliter was determined by plating 10-fold dilutions on Luria agar. Each datum point represents the mean of triplicate wells from a single experiment. Bars represent geometric means.

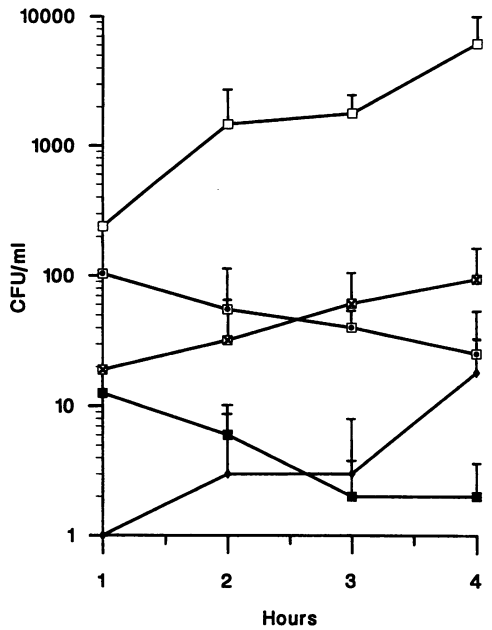

FIG. 3. Internalization of *P. mirabilis* strains by HRPTEC over time. Suspensions of bacterial strains (10^8 CFU) were overlaid onto monolayers of HRPTEC and incubated at 37°C. At 1, 2, 3, and 4 h, monolayers were treated with gentamicin and washed, and HRPTEC were lysed with Triton X-100. The number of CFU of lysate per milliliter was determined by plating 10-fold dilutions of the suspension on Luria agar. Datum points and bars represent means and standard deviations of three experiments with triplicate determinations for each experiment. For the comparison between *P. mirabilis* BA6163 (hemolytic) and WPM111 (hemolysin-negative mutant), $P = 0.64$, 0.068, 0.0014, and 0.031 for 1, 2, 3, and 4 h, respectively, when values were compared by the *t* test. Symbols: □, *S. typhimurium*; ▫, *P. mirabilis* HI4320; ■, *P. mirabilis* BA6163; □, *P. mirabilis* WPM111; ◆, *E. coli*.

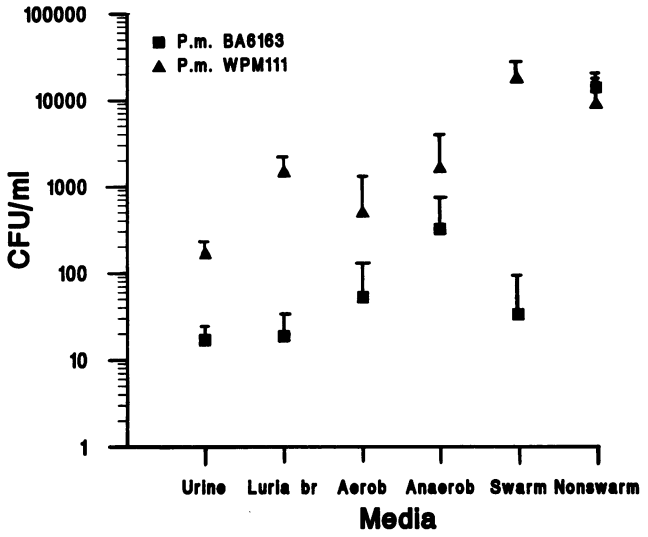

FIG. 4. Effect of HpmA hemolysin on internalization of *P. mirabilis* (P.m.). Suspensions of *P. mirabilis* BA6163 and its HpmA hemolysin-negative mutant, grown under six growth conditions, were overlaid onto HRPTEC monolayers and incubated for 3 h at 37°C. Viable counts of gentamicin-protected bacteria were determined as described in Materials and Methods. Hemolytic titers for strain BA6163 grown under each culture condition are given in parentheses: Luria broth (1:4), urine (1:2), aerobic (1:4), anaerobic (1:2), edge of swarm (1:32), and nonswarming (1:4).

by light microscopy and demonstrated clearly by transmission electron microscopy of thin sections of the fixed kidney cells (Fig. 1). Bacteria were only very rarely observed to be free in the cytoplasm, suggesting that the principal pathway of internalization results in enclosure of bacteria in vacuoles. The internalization of the bacteria by HRPTEC was corroborated by using the gentamicin protection assay, in which bacteria that gain entry to the eucaryotic cell are not susceptible to the antibiotic (22) when it is added to the tissue culture medium (Fig. 2). *P. mirabilis* strains, as a group, were internalized, but less so than *S. typhimurium*, a well-established intracellular pathogen (22). The lack of inhibition of this process by cytochalasin D suggests that microfilament formation (6) is not involved in the mechanism by which *P. mirabilis* gains access into these cultured renal cells. This lack of inhibition has also been observed for a urinary tract isolate of *Citrobacter freundii* overlaid onto a T24 bladder cell line (15).

A major limitation of the gentamicin assay for quantitating bacteria internalized by HRPTEC was the cytolytic effect of the pore-forming HpmA hemolysin produced by all strains of *P. mirabilis* (13, 16, 18, 24, 25). Incubation of strains with HRPTEC resulted in cell lysis and complete clearing of the monolayers. Bacterial cells that had been internalized could have been released or at least exposed to gentamicin when the integrity of the cell was compromised by the action of HpmA hemolysin.

That HpmA hemolysin is responsible for this cytolysis is evident from specific observations. The hemolytic titer of *P. mirabilis* strains correlates with monolayer destruction. The higher the titer, the more rapidly the monolayer is cleared. For example, when *P. mirabilis* was grown under conditions known to enhance HpmA expression (i.e., agar-grown bacteria harvested from the edge of an active swarm [1, 2, 4, 9]), bacteria lysed HRPTEC monolayers more rapidly than when bacteria were grown statically in broth, overnight with shaking, or under

TABLE 2. Effect of cytochalasin D on internalization of *P. mirabilis* by HRPTEC

Strain	CFU/ml recovered after gentamicin treatment[a] at given cytochalasin concn[b]	
	0 μM	20 μM
P. mirabilis BA6163	70 ± 100	40 ± 60[c]
P. mirabilis WPM111	280 ± 370	470 ± 420[c]
S. typhimurium	18,600 ± 8,300	1,900 ± 700[d]
E. coli HB101	0	1 ± 2[c]

[a] Bacteria cultured in Luria broth under static conditions were incubated with HRPTEC for 3 h prior to gentamicin addition. Results are expressed as mean ± standard deviation of three separate experiments, each with triplicate determinations.
[b] HRPTEC were preincubated for 30 min with cytochalasin D.
[c] $P > 0.1$ compared with no cytochalasin pretreatment.
[d] $P < 0.05$ compared with no cytochalasin pretreatment.

anaerobic conditions. In bacteria isolated from the swarm edge, however, there was the possibility that additional, as yet uncharacterized, cytolysins were expressed under this culture condition. To clarify this point, the isogenic HpmA hemolysin-negative mutant strain (WPM111) constructed by Swihart and Welch (24, 25) was tested and found not to lyse HRPTEC and not to cause clearance of the monolayer.

In a previous report, Peerbooms et al. (17) correlated *P. mirabilis* invasion of Vero cells with hemolysin production. They examined 32 urinary strains and showed that internalization into Vero cells, as measured by an aminoglycoside protection assay, increased proportionally to the hemolytic titers of the strain. A chemically induced mutant with elevated hemolysin production invaded Vero cells in numbers 5- to 10-fold higher than the parent strain. When hemolysin (and other protein) production was inhibited by chloramphenicol, invasion was reduced by 20%. Our data contradict these findings and indeed could lead us to the opposite conclusion, that is, that hemolysin activity reduces the magnitude of invasion. Our data, however, demonstrate that wild-type *P. mirabilis* may be internalized but simply is not protected from the action of gentamicin because of hemolysin-induced pore formation and/or complete cytolysis of HRPTEC. When hemolysin is not expressed (strain WPM111), bacteria are internalized and protected from gentamicin, thus elevating the number of recoverable bacteria. While we cannot rule out the possibility that swarming bacteria have invaded within the first few minutes in high numbers and destroyed the HRPTEC from inside, we speculate that hemolysin works from outside the eucaryotic cell. Possible explanations for the discrepancy between our work and previous reports (17, 19) may be the differential sensitivity of Vero cells and HRPTEC to the HpmA hemolysin or the presence of uncharacterized mutations induced by chemical mutagenesis.

Also in sharp contrast to our findings, Allison and colleagues (1) reported that elongated swarm cells were internalized in significantly higher numbers than the corresponding vegetative (short, rod-shaped) bacteria. While they also observe that hemolytic activity is markedly increased in swarm cells, they do not report lysis of their monolayers of the "urothelial" transformed cell line. It is possible that this transformed cell line is resistant to cytolysis by the HpmA hemolysin or even that the swarm cells added to the monolayers were more resistant to the killing of the gentamicin added during the aminoglycoside protection assay.

These authors further suggest that swarm cells invade the

urothelial cell, maintaining their differentiated appearance, and multiply freely in the cytoplasm. In contrast to their results, we find that the short, rod-shaped, vegetative form of *P. mirabilis* is internalized within membrane-bound vacuoles and does not undergo any significant replication (data not shown). Indeed, when active swarm cells are added to HRPTEC monolayers, they rapidly revert to short rods and are internalized in that form. Again, these differences may reflect the use of primary cultures of human renal epithelial cells described in this report versus transformed cell lines described previously (1). Although we did not examine this aspect in as much detail, our observations suggest that when swarming differentiation is inhibited by culture conditions greater internalization, rather than less, occurs.

We have observed *P. mirabilis* bacterial cells within membrane-bound vacuoles in cultured renal cells. Conditions in which large numbers of these bacteria were internalized by the HRPTEC were optimized. A deletion mutant lacking hemolysin provided even higher levels of invasion. These observations represent in vitro results, however, and may or may not reflect the events that occur in natural or experimental infection. We must next look carefully to the renal cells of experimentally infected animals for evidence that the cells of the intact host can or cannot internalize *P. mirabilis.*

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