Ability of *Proteus mirabilis* To Invade Human Urothelial Cells Is Coupled to Motility and Swarming Differentiation

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Proteus mirabilis causes serious kidney infections which can involve invasion of host urothelial cells. We present data showing that the ability to invade host urothelial cells is closely coupled to swarming, a form of cyclical multicellular behavior in which vegetative bacteria differentiate into hyperflagellated, filamentous swarm cells capable of coordinated and rapid population migration. Entry into the human urothelial cell line EJ/28 by *P. mirabilis* U6450 isolated at different stages throughout the swarming cycle was measured by the antibiotic protection assay method and confirmed by electron microscopy. Differentiated filaments entered urothelial cells within 30 min and were 15-fold more invasive (ca. 0.18% entry in 2 h) than an equivalent dry weight of vegetative cells isolated before differentiation, which attained only ca. 0.012% entry in the 2-h assay. The invasive ability of *P. mirabilis* was modulated in parallel with flagellin levels throughout two cycles of swarming. Septation and division of intracellular swarm cells produced between 50 and 300 vegetative bacteria per human cell, compared with 4 to 12 intracellular bacteria after incubation with vegetative cells. Transposon (Tn5) mutants of *P. mirabilis* with specific defects in motility and multicellular behavior were compared with the wild-type for the ability to invade. Mutants which lacked flagella (nonmotile nonswarming) were entirely noninvasive, and those which were motile but defective in swarm cell formation (motile nonswarming) were 25-fold less invasive than wild-type vegetative cells. Mutants with defects in the coordination of multicellular migration and the temporal control of consolidation (cyclical reversion of swarm cells to vegetative cells) were reduced ca. 3- to 12-fold in the ability to enter urothelial cells. In contrast, a nonhemolytic transposon mutant which swarmed normally retained over 80% of wild-type invasive ability. Swarm cells and early consolidation cells were at least 10-fold more cytolytic than vegetative cells as a result of their high-level production of hemolysin.

Urinary tract infections caused by *Proteus mirabilis* are common and often severe, leading to acute pylonephritis, chronic inflammation, periurethral abscesses, renal failure, and bacteremia (7, 19, 40, 44). About 90% of *P. mirabilis* urinary tract infections show renal involvement, and this predilection for the upper urinary tract has been confirmed in animal models, which have also shown that *P. mirabilis* is able to invade host kidney cells (6, 36, 38, 40). *P. mirabilis* has a number of putative virulence factors, including the secreted hemolytic toxin HpmA, which has been suggested to contribute to host cell invasion and cytotoxicity (32, 37, 38, 43), an inducible urease which, by generating ammonia, causes precipitation of bladder and kidney stones (6, 30), fimbriae which promote bacterial adherence to the uroepithelium (4, 41), a secreted protease able to digest immunoglobulins (28), and the as yet unidentified determinants that facilitate invasion of mammalian kidney cells (38). The invasion of host cells by *P. mirabilis* during infection can be readily assayed by using immortalized mammalian cell lines cultured in vitro (3, 6, 38).

Also in contrast to other uropathogens, *P. mirabilis* exhibits a form of multicellular behavior known as swarming migration, a cyclical differentiation process in which typical vegetative rods (2 to 4 μm long) undergo differentiation at the colony margin into long (up to 80 μm), multinucleate, aseptate filaments that possess up to 50-fold more flagella per unit cell surface area (2, 3, 17, 49). These swarm cells migrate rapidly and coordinately away from the colony until they stop and revert by cell division into the short vegetative cell form, a process termed consolidation. The swarming process continues as periodic cycles of cell differentiation, population migration, and consolidation. It seems reasonable to suppose that the ability to differentiate into a population of cells capable of rapid surface migration might have a role in renal infections, which normally involve colonization of the lower urinary tract followed by ascending migration of bacteria against the flow of urine and mucus (40). Indeed, experiments with blocking antibodies have shown that flagella are required for kidney infection by *P. mirabilis* (34, 35) and that motility is essential for *P. mirabilis* to infect burn wounds (16). The recent isolation of *P. mirabilis* transposon insertion mutants with specific defects in motility, differentiation, and migration has opened the way to an assessment of the hitherto undefined importance of swarming behavior in pathogenicity (1, 3, 5). In this study, we present evidence that the ability of *P. mirabilis* to invade human urothelial cells is closely associated with motility and is modulated during the swarming cycle, being primarily an attribute of differentiated swarm cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. mirabilis* U6450 was isolated from a chronic urinary tract infection involving renal stone formation (25). It is of proticine type P3/S1,13 and has a strong cell-associated hemolytic activity. *Escherichia coli* LE2001 was provided by B. Holland, Leicester University, Leicester, United Kingdom. *P. mirabilis* U6450 was grown at 37°C in Luria broth (LB) and on 2% LB agar.

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Petri plates (9-cm diameter) containing LB agar were centrally inoculated with overnight LB cultures of wild-type *P. mirabilis*. At timed intervals, sections of agar containing fresh vegetative cells (central colony and consolidation zones) and swarm cells (colony edge) were cut aseptically from plates inoculated in parallel, and bacteria were washed from agar segments with phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 0.9% [wt/vol] sodium chloride [pH 7.2]).

Dry weight determination and viable counts. Two methods were used to normalize bacterial suspensions used for invasion assays. Separate standard curves for bacterial dry weight against A_{600} were constructed (24) by using serially diluted fresh swarm cell and vegetative cell suspensions, and the linear region of each curve was used to estimate bacterial dry mass. One absorbance unit was roughly equivalent to 0.6 mg (dry weight) of cells per ml for both vegetative and differentiated bacteria. To estimate total viable cell units, suspensions of bacteria in 0.9% saline were allowed to stand at 24°C for 4 h to allow reversion of swarm cells to the constituent vegetative cells prior to serial dilution and estimation of viable cell numbers by plating on MacConkey agar.

Isolation of transposon mutants. Mutations were introduced into the chromosome of the spontaneous rifampin-resistant mutant of *P. mirabilis* U6450 by using the Tn5-based transposon Tn*phoA*. Swarm-defective transposon insertion mutants were characterized according to motility, flagella production, and swarm cell formation (1). Kanamycin-resistant nonhemolytic mutants were identified by replica plating transconjugants into wells of microtiter plates containing LB broth, which were incubated for 3 h at 37°C prior to addition of a horse erythrocyte suspension in saline and a further 30-min incubation. Nonhemolytic transconjugants were visualized as wells containing no soluble hemoglobin (red color) in the supernatant following centrifugation at 2,000 × *g* for 10 min.

Mammalian cell culture. The African green monkey kidney epithelial cell line Vero and two human urothelial cell lines, EJ/28 and 5637, were obtained from the Imperial Cancer Research Fund laboratories, London, United Kingdom. The human cell lines were fully transformed human uroepithelial cells, each derived from a transitional cell carcinoma of the urinary bladder. Cells were routinely maintained and passaged twice weekly in Glasgow's modified Eagle's medium containing 10% fetal calf serum (Sera Lab) and 2 mM glutamine at 37°C in a humidified 10% carbon dioxide atmosphere.

Invasion and cytotoxicity assays. Culture plate wells containing confluent monolayers of cells were washed twice with Hanks balanced salts solution (HBSS) before application to each well of 1 ml of a bacterial suspension normalized to bacterial dry weight and containing ca. 5 × 10⁷ bacterial cell units per ml in incubation solution (HBSS-minimal medium [10]-0.2 M Tris buffer [pH 7.5], 80%:10%:10% [vol/vol]) and incubation at 37°C under an atmosphere of 10% CO₂. At timed intervals (2 h for the standard assay), bacterial suspensions were removed, assessed for mean cell length (MCL), and serially diluted in incubation solution prior to determination of the viable bacterial titer on MacConkey agar. Urothelial cells were washed twice with HBSS, 1 ml of Glasgow's modified Eagle's medium containing streptomycin sulfate (250 µg/ml) was added, and the plates were incubated for a further 1.5 h at 37°C. Cell monolayers were washed twice with HBSS, and their integrity was determined by light microscopy. They were then lysed by incubation for 30 min at 37°C with 1 ml of lysis solution (1% [vol/vol] Tween 20–0.025% [wt/vol] trypsin in 0.01 M sodium phosphate buffer [pH 8.0]). Cell lysates were serially diluted in 0.9% saline, and viable bacteria were counted as described above. All experiments were carried out at least twice, using triplicate wells for each bacterial suspension, and results were expressed as percent total inoculum viable counts that survived the streptomycin treatment. To determine cytotoxicity, serially diluted bacteria were applied to the surface of cell monolayers in culture plates in triplicate as described above; after incubation for 2 h, the number of intact urothelial cells remaining in each well was estimated by light microscopy. Results were expressed as bacterial numbers causing ca. 50% cell lysis. Noninvasive, nonhemolytic (HlyA⁻) and hemolytic (HlyA⁺) *E. coli* strains which were noncytotoxic and roughly equivalent in cytotoxicity to the wild-type *P. mirabilis* vegetative cells, respectively, were used as assay controls. Variation of 10 to 20% in cytotoxicity and invasion data was commonly observed between experiments for the same bacterial concentration, but within each experiment, results were highly reproducible (standard errors of the means < 6% of means). Mean data were compared by using the Student *t* test.

Microscopy. Bacterial suspensions were routinely assessed for the extent of differentiation by phase-contrast light microscopy, and MCL was estimated by reference to a ruled grid on a calibrated microscope slide. Monolayers of EJ/28 cells, grown on glass coverslips and fixed in 2% paraformaldehyde, were permeabilized in 0.1% Triton X-100, incubated with monoclonal anti-human keratin primary antibodies (Sigma Chemical Co.), and labeled with fluorescein-conjugated antibodies for indirect immunofluorescence as described previously (14). Confocal microscopy was performed by using a Bio-Rad MRC 500 system. For transmission electron microscopy, cell monolayers grown in 24-well plastic culture plates were washed with PBS and fixed in 2% glutaraldehyde–0.1 M sodium phosphate buffer (pH 7.4) overnight. Cells were scraped from the plastic surface prior to postfixing in 1% OsO₄ in 0.1 M phosphate buffer for 90 min and staining with 0.25% uranyl acetate overnight. Samples were dehydrated in an ethanol series and embedded in firm Spurr's resin prior to sectioning and staining with uranyl acetate and lead citrate. Bacterial suspensions were stained for transmission electron microscopy with phosphotungstate (1).

Assay of hemolysin. Cell-bound hemolytic activity was assayed in bacterial suspensions in PBS as described previously (25) and expressed as units (1 U of activity caused the release of 50 mg of hemoglobin per h in the standard assay). Activities were normalized to bacterial dry weight as described previously (24).

Flagellin determination. Flagella were isolated from fresh bacterial suspensions washed from the agar surface in PBS by vigorous vortexing for 2 min followed by centrifugation at 15,600 × *g* to remove cells. Flagellin in the supernatant was concentrated by trichloroacetic acid precipitation (10% [wt/vol]) at 4°C, resuspended in 20 µl of sodium dodecyl sulfate (SDS) sample buffer, fractionated on 12.5% polyacrylamide gels containing 0.1% SDS, and stained with Coomassie brilliant blue (26). Protein content of stained flagellin bands, normalized to bacterial dry weight (24), was quantified by photographing gels before scanning the photographic negatives on a Joyce-Loebl Chromoscan3 densitometer.
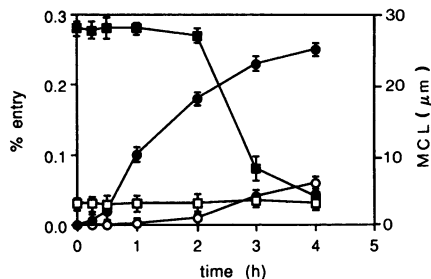
FIG. 1. Invasion into human urothelial cells by vegetative and swarm cells of wild-type *P. mirabilis* U6450. Fresh suspensions of either vegetative cells or swarming filaments containing ca. 10^7 cell units per ml were normalized to A_{600} before incubation with monolayers of urothelial cells. Invasion was assayed by the streptomycin protection method and expressed as percentage of total viable counts that survived incubation with the antibiotic. MCL of bacterial populations was measured throughout the time course. Values are means of three determinations; bars represent standard errors of the means. Symbols: open, vegetative cells; closed, swarm cells; squares, MCL; circles, percent entry.

RESULTS

Invasion of urothelial cells is primarily a trait of motile and differentiated *P. mirabilis*. We assayed a monkey kidney parenchyma (Vero) cell line previously used in *P. mirabilis* invasion assays (38) and two different human urothelial cell lines and found them to be roughly equivalent in susceptibility to invasion and cytolysis by *P. mirabilis* (data not shown). For subsequent assays, the human uroepithelial cell line EJ/28 was chosen for its relevance as a target cell in *P. mirabilis* renal infection.

To determine the relative invasive ability of vegetative cells and differentiated filaments of *P. mirabilis*, entry into urothelial cells was monitored over a 4-h time course, using fresh bacterial suspensions normalized for cell unit number (dry weight equivalents) by measurement of A_{600}. Extracellular bacteria were killed by incubating monolayers with streptomycin (which cannot kill intracellular bacteria but killed >99.99% of extracellular bacteria) prior to osmotic lysis of the urothelial cells and enumeration of the viable bacteria released. Results showed that invasion by swarm cells occurred within 30 min and was about 15-fold greater (ca. 0.18% entry) after 2 h than was invasion by vegetative cells (ca. 0.012% entry), which were internalized more slowly (Fig. 1). MCL of *P. mirabilis* suspensions was monitored by phase-contrast light microscopy in parallel with invasion assays during the 4-h time course. Results showed that cell division of differentiated filaments (MCL, 28.5 µm) to vegetative cells occurred primarily after 2 h of incubation with urothelial cells and was almost complete after 3 h, whereas the MCL of vegetative cell populations (ca. 2.7 µm) did not change significantly during the experiment (Fig. 1).

Observation of the invasion process by confocal microscopy of urothelial cells infected for 1 h with differentiated swarm cells and stained with monoclonal anti-human keratin antibodies, which reacted strongly with *Proteus* cells as well as the urothelial cytokeratin, showed many apparently intracellular filamentous bacterial cells aligned with the nuclear membrane and throughout the cytoplasm (Fig. 2A). The intracellular location of these bacteria was confirmed by transmission electron microscopy, which demonstrated that more than 90% of visible bacteria were surrounded by urothelial cell cytoplasm. At higher magnification, a light-
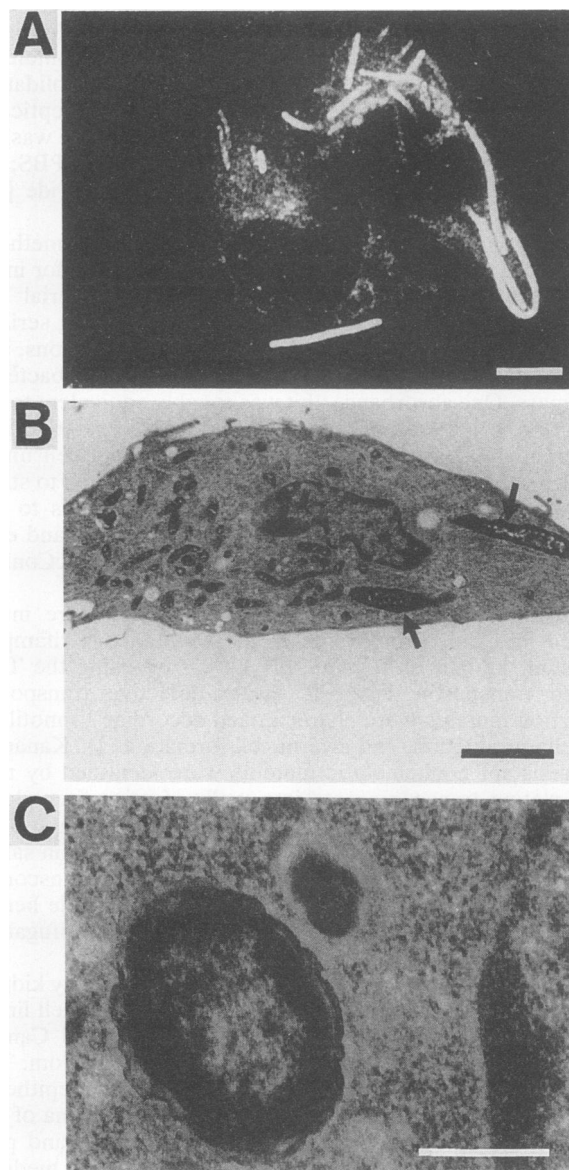

FIG. 2. (A) Confocal optical section of a subconfluent monolayer of cultured EJ/28 urothelial cells infected for 1 h with *P. mirabilis* swarm cells and stained by indirect immunofluorescence with monoclonal antikeratin antibodies. The section shown is the eighth in a series of 20 taken at 0.6-µm intervals moving upward from the basolateral region of the cell margin toward the apical surface. Bacteria were nonspecifically stained along with the cytokeratin, which forms a diffuse outline of intermediate filaments within the urothelial cell. Bar, 5 µm. (B) Corresponding transmission electron micrograph of a section through an infected urothelial cell. Arrows mark internalized bacteria. Bar, 5 µm. (C) Transmission electron micrograph of a section through an infected urothelial cell showing detail of internalized *P. mirabilis*. Bar, 0.5 µm.

staining area was apparent between the bacterial cell wall and the dark-staining urothelial cell cytoplasm, possibly suggesting that bacteria were contained within vacuoles, although vacuolar membranes were not clearly visible (Fig. 2C). After 2 h, intracellular filaments began to septate and divide into vegetative cells, and at 3 h, each urothelial cell contained between 50 and 300 short rods distributed

swarming cycle

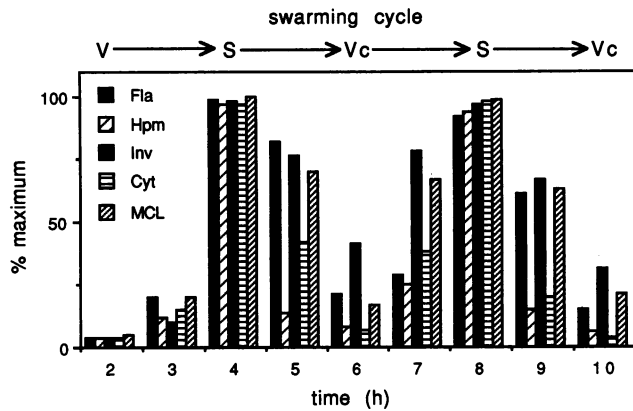V ———→ S ———→ Vc ———→ S ———→ Vc

FIG. 3. Differential invasion into urothelial cells (Inv), cytotoxicity (Cyt), hemolysin activity (Hpm), flagellin production (Fla), and MCL of *P. mirabilis* during two swarming cycles. Bacteria were inoculated centrally onto parallel LB agar plates, and fresh cells were removed from the colony during vegetative growth (V), during migration of differentiated swarm cells (S), and following consolidation to vegetative cells (Vc). Results are means of three determinations, and units are calculated relative to maximal values for each characteristic.

throughout the cytoplasm and into the cytoplasmic cell extensions (data not shown).

Assays of urothelial cell invasion by *P. mirabilis* performed with suspensions of swarming stage-specific bacterial cells isolated throughout two swarming cycles and normalized for bacterial dry weight showed that the ability to enter urothelial cells was modulated during the swarming cycle in parallel with the differential expression of flagellin, the most obvious marker of differentiation (Fig. 3). In particular, differentiated swarm cells and cells in the early consolidation stages of swarming were 15- to 20-fold more invasive than vegetative cells grown in liquid broth (data not shown) or vegetative cells isolated from the center of swarming colonies when compared directly in terms of dry weight, i.e., bacterial cell units. Swarming populations were always more invasive than the colony core vegetative cells isolated prior to differentiation, since consolidated vegetative cells

retained about 40% of the swarm cell invasive ability (Fig. 3).

Mutants with defects in motility and swarming are less able to invade urothelial cells. We have previously identified five separate swarm-defective phenotypes of *P. mirabilis* following Tn5 insertion mutagenesis: nonmotile nonswarming (NMNS), motile nonswarming (MNS), dendritic swarming (DS), and infrequent and frequent consolidation (IC and FC). NMNS mutants lack functional flagella; MNS mutants have flagella and are motile but are unable to differentiate into swarm cells. DS mutants display an irregular form of swarming during which consolidation frequency is approximately the same as in the wild type but in which the migrating fringe is filamentous and zones of consolidation are irregular. Insertion mutations which cause defects in the temporal control of migration give rise to infrequent and frequent consolidation (IC and FC phenotypes). All Tn5 mutants capable of swarming formed the hyperflagellated swarm cells characteristic of the wild type, and flagellum production appeared identical to that in the wild type in all mutants except the nonflagellated NMNS mutants (Table 1).

Invasion assays carried out as before, using vegetative cell suspensions of two independently isolated Tn5 mutants from each swarm-defective phenotypic class, showed that Tn5 insertions causing defects in motility and swarming behavior had profound effects on the ability of *P. mirabilis* to invade human EJ/28 urothelial cells (Table 1). Mutants within the same swarm-defective phenotypic class exhibited closely similar levels of invasion. The nonflagellated NMNS mutants were completely noninvasive, identical to the noninvasive control, *E. coli* LE2001, whereas MNS and DS mutants had very limited invasive ability (4 and 8%, respectively, of wild-type invasion). FC and IC mutants that were defective in the temporal control of consolidation frequency also exhibited considerably reduced invasion (10 and 28%, respectively, of wild-type invasion) (Table 1).

To test the requirement of flagellin protein for invasion by *P. mirabilis*, a crude preparation of sheared swarm cell flagella was added to mutant bacterial suspensions (final flagellin concentration, ca. 2 mg/ml) prior to invasion assays. The addition of flagellin had no significant effect on the invasive ability of any mutant phenotype (data not shown).

TABLE 1. Invasive, cytotoxic, and hemolytic activities of wild-type and mutant *P. mirabilis*

P. mirabilis strain	Motility	Swarm cells	Relative values[a]			Swarming phenotype
			Invasion[b]	Cytotoxicity[c]	Hemolysin activity[d]	
U6450	+	+	100	100	100	Wild type
NMNS8	−	−	<0.2	98	97	Nonswarming
NMNS33	−	−	<0.2	100	100	
MNS15	+	−	4.5	115	133	Nonswarming
MNS7	+	−	3.7	100	103	
DS35	+	+	8.6	97	100	Dendritic swarming
DS24	+	+	7.9	99	108	
FC18	+	+	10.2	102	110	Frequent consolidation
FC28	+	+	10.6	100	100	
IC32	+	+	30.0	100	97	Infrequent consolidation
IC37	+	+	27.8	105	103	
NH1	+	+	82.0	<0.1	<0.1	Wild type

[a] Values are means of three determinations (standard error of the mean <6% of means) and are normalized to the wild-type vegetative cell level (100%).

[b] The actual percentage of vegetative wild-type *P. mirabilis* that entered urothelial cells was 0.012% ± 0.0008%. The invasive ability of each mutant was significantly lower than that of wild-type *P. mirabilis* ($P < 0.01$).

[c] The wild-type *P. mirabilis* vegetative cell cytotoxicity titer causing 50% urothelial cell lysis was 8×10^7 viable bacteria per ml.

[d] The hemolytic activity of vegetative wild-type *P. mirabilis* cells was 378 U/mg (dry weight).

Cell-associated hemolysin Pmh determines cytotoxicity but not invasion. Levels of secreted hemolysin activity underwent dramatic changes during the swarming cycle, being highest in swarm cell populations (Fig. 3). Wild-type swarm cells were approximately 18-fold more cytotoxic to urothelial cells than were wild-type vegetative cells when compared on a dry weight basis (Fig. 3) and were at least 10^3 times more cytotoxic, again per unit of dry weight, than either vegetative cells or swarm cells of the nonhemolytic transposon mutant NH1 (Table 1). Incubation of urothelial cell monolayers with up to 3×10^6 wild-type swarm cells per ml caused no detectable urothelial cell lysis within the 2-h assay. Invasion assays carried out in parallel with the nonhemolytic *P. mirabilis* NH1 showed it to be almost as invasive (82%) as the wild type at bacterial concentrations which did not permit urothelial cell lysis. The number of intracellular *P. mirabilis* NH1 increased concomitantly with bacterial concentration in the standard 2-h assay up to a threshold inoculum concentration of 5×10^9 bacteria per ml, when urothelial cell lysis became evident (data not shown).

DISCUSSION

We have assessed the degree to which swarming differentiation and motility are related to an apparently important aspect of *P. mirabilis* uropathogenicity, the ability to enter mammalian urothelial cells. Swarming forms of *Proteus* spp. have not been reported in vivo, but it seems likely that rapid population migration would be an asset in establishing ascending infection of the urinary tract and when in the human kidney the bacteria must traverse the single cell layer of the urothelium in order to infect the underlying renal parenchyma (40). Peerbooms and coworkers (38) demonstrated invasion of Vero cells by vegetative *P. mirabilis*, but there has been no report of the relationship between swarming and invasion. Our data obtained with human EJ/28 urothelial cells confirm that invasion is directly related to incubation time and bacterial concentration and show that invasion is indeed primarily a trait of motile differentiated cells. They support the view that significant intracellular bacterial growth does not occur following invasion by vegetative cells, although internalized swarm cells do, after a time, septate and divide into vegetative rods, generating between 4 and 28 vegetative bacteria per invading filament. In addition to establishing that the swarming cycle is characterized by dramatic changes in the ability of *P. mirabilis* to invade uroepithelial cells, we show that specific mutations affecting motility, differentiation, and to a lesser extent multicellular migration significantly reduce bacterial invasion into urothelial cells. Our data indicate strongly that the differentiated swarm cell filaments of *P. mirabilis* are an invasive cell form which may play a major role in colonization of human epithelia during urinary tract infections. Invasion into human urothelial cells by *P. mirabilis* vegetative cells in this study (ca. 0.01%) was 50- to 100-fold greater than levels reported previously for *P. mirabilis* entry into Vero cells (38), but even swarm cell levels of invasion (ca. 0.2%) were low compared with invasion by the highly invasive strains of *Salmonella* and *Yersinia* spp., which are reported to achieve up to 50% entry into eukaryotic cells (22).

Hemolysin (HpmA) production has previously been implicated in the pathogenesis of *P. mirabilis* urinary tract infections (37, 43) and specifically as a factor determining the invasion of eukaryotic cells (38), but its involvement in infection is unclear, as a recent study of HpmA-negative *P. mirabilis* mutants has suggested only a minor role for this

toxin in an experimental murine model of ascending tract pyelonephritis (45). In our study, the nonhemolytic mutant was only slightly less able to invade eukaryotic cells than was the wild type.

Microscopy of *P. mirabilis* invasion into urothelial cells indicated that differentiated swarm cells were taken up in greater numbers than were vegetative cells, and once the cells were bound to or internalized by the urothelial cell, septation of the filament was repressed for up to 1 h in comparison with free-floating, unattached bacteria, which began to divide into short cells within 40 min of incubation. These data suggest that swarm cells of *P. mirabilis* may detect their proximity to the mammalian cell membrane and that this may influence their rate of reversion to vegetative cells. The ability to differentiate between life on a surface and life in a liquid environment is an established feature of swarming behavior in *Vibrio parahaemolyticus*. This bacterium uses its single polar flagellum as a dynometer to initiate swarming in viscous environments by transducing a signal to swarm, which in turn induces transcription of genes directing the synthesis of the many lateral flagella that are required for swarming motility (29). The initial interaction of a number of invasive bacteria with the eukaryotic cell surface has also been shown to be accompanied by cell-cell signalling; e.g., in *Salmonella typhimurium*, specific changes in gene expression are induced by contact with epithelial cell surfaces (47).

The complete loss of invasive ability by nonmotile *P. mirabilis* mutants in this study suggests an intrinsic requirement for flagella or flagellum-mediated motility in invasion into urothelial cells; this finding is perhaps not surprising, as motility is an invasion-related factor in a number of pathogenic bacteria, such as *Campylobacter jejuni*, *Salmonella typhi*, and *Vibrio cholerae* (27, 39, 46). However, some nonmotile bacteria, such as *Shigella flexneri*, are able to invade eukaryotic cells (8), and our finding that motile, swarm-defective mutants of *P. mirabilis* also exhibit significantly reduced invasive ability is consistent with the involvement of additional bacterial factors in the invasion process. Furthermore, in this study, extracellular complementation of noninvasive swarm-defective *P. mirabilis* mutants was not achieved by adding crude flagellin preparations to bacterial suspensions prior to invasion assays, suggesting that flagellin per se does not determine bacterial uptake by urothelial cells. In other bacteria, differentiation and multifactorial virulence processes often require the coordinate expression of multiple genes under the control of central transcriptional activators (30). It is possible that a regulatory locus is affected in some of our swarm-defective *Proteus* mutants, leading to altered expression of genes required for both differentiation and invasion. A recent study of *S. typhimurium* mutants indicated that both motility and chemotaxis were required for invasion into mammalian cells, with the ability to rotate flagella counterclockwise essential for bacterial entry into the eukaryotic cells (23). An early study of nonswarming and nonchemotactic mutants of *P. mirabilis* concluded that chemotaxis was unlikely to play a major role in swarming (48), but the recent observation that some swarm-defective *P. mirabilis* transposon mutants also exhibit defects in chemotaxis has suggested that at least some components may be common to the swarming and chemotaxis systems (5).

Extensive studies of invasion into eukaryotic cells by a number of enterobacteria and the gram-positive bacterium *Listeria monocytogenes* have led to identification and characterization of bacterial cell surface proteins which mediate

entry of bacteria into cultured mammalian cells, such as invasin of *Yersinia pseudotuberculosis* and internalin of *L. monocytogenes* (12, 15, 22). Invasion-related surface proteins of *P. mirabilis* have not been identified, but recently Moayeri and coworkers (31) have demonstrated that immunization with purified outer membrane proteins of *P. mirabilis* offers protection for mice in an experimental pyelonephritis model. Furthermore, it has been suggested that the affinity by which bacteria such as *Yersinia* organisms bind to eukaryotic cells is a major factor influencing bacterial uptake (20). Swarm cell filaments which are composed of multiple cell units may exhibit stronger binding affinity than do single vegetative rods as a result of the presence of more putative binding ligands per bacterial cell.

The delayed septation and division of swarm cells associated with mammalian cells may reflect a bacterial response to altered physiological conditions such as pH and nutrient availability or, alternatively, indicate specific recognition processes involving binding of surface proteins of *P. mirabilis* to urothelial cell surface molecules. Candidate host proteins may be members of the $\beta 1$-chain integrin family which act as receptors for *Y. pseudotuberculosis* invasin and surface proteins that mediate the entry of several other microorganisms into epithelial cells (12, 21). A number of biochemical and physiological factors are reported to be modulated during the swarming cycle (2, 9, 11, 18, 49), and our recent data show that high levels of flagellin, hemolysin, and urease production in swarming cells are due to specific transcriptional activation (3). If the ability of *P. mirabilis* to enter eukaryotic cells is indeed determined by a bacterial cell surface invasin-like protein, this protein may be synthesized differentially during the swarming cycle, for example, as a result of modulation of gene expression.

ACKNOWLEDGMENTS

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