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Invasion of Cultured Human Epithelial Cells by *Klebsiella pneumoniae* Isolated from the Urinary Tract

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The mechanisms which enable entry into cultured human epithelial cells by *Klebsiella pneumoniae* were compared with those of *Salmonella typhi* Ty2. *K. pneumoniae* 3091, isolated from a urine sample of a patient with a urinary tract infection, invaded human epithelial cells from the bladder and ileocecum and persisted for days in vitro. Electron microscopic studies demonstrated that *K. pneumoniae* was always contained in endosomes. The internalization mechanism(s) triggered by *K. pneumoniae* was studied by invasion assays conducted with different inhibitors that act on prokaryotic and eukaryotic cell structures and processes. Chloramphenicol inhibition of bacterial uptake revealed that bacterial de novo protein synthesis was essential for efficient invasion by *K. pneumoniae* and *S. typhi*. Interference with receptor-mediated endocytosis by g-strophanthin or monodansylcadaverine and inhibition of endosome acidification by monensin reduced the number of viable intracellular *K. pneumoniae* cells, but not *S. typhi* cells. The depolymerization of microfilaments by cytochalasin D inhibited the uptake of both bacteria. Microtubule depolymerization caused by colchicine, demecolcine, or nocodazole and the stabilization of microtubules with taxol reduced only the invasion ability of *K. pneumoniae*. *S. typhi* invasion was unaffected by microtubule depolymerization or stabilization. These data suggest that the internalization mechanism triggered by *K. pneumoniae* 3091 is strikingly different from the solely microfilament-dependent invasion mechanism exhibited by many of the well-studied enteric bacteria, such as enteroinvasive *Escherichia coli*, *Salmonella*, *Shigella*, and *Yersinia* strains.

Klebsiella pneumoniae, a widely recognized opportunistic pathogen that causes nosocomial pneumonia, bacteremia, and septicemia, is also involved in urinary tract infections (UTI). In a recent study, *K. pneumoniae* was the second species, after *Escherichia coli*, most frequently isolated from more than 400 women with recurrent UTI (45). Treatment of *K. pneumoniae* infections has become increasingly difficult because of the predominance of multiple-antibiotic-resistant strains (1, 5, 22). Resistance to broad-spectrum cephalosporins has also been reported with increasing frequency (6).

Several factors are thought to be important in the pathogenesis of *K. pneumoniae*. The ability of *K. pneumoniae* to colonize human respiratory and urinary epithelia may be due to type 1, 3, or 6 pili (singly or in combination) or to a nonfimbrial protein adhesin (6, 12). Adherence is also important for colonization of the intestine, the suspected reservoir for UTI (43). Aerobactin production enhances virulence (29); complement resistance and bacterial proliferation in the host depend on the synthesis of capsular polysaccharide (26). Finally, toxin production by *K. pneumoniae* has been shown to be lethal to mice (47).

The occurrence of *Klebsiella* invasion in vivo has not yet been investigated and, therefore, remains unproven as another factor that contributes to the pathogenicity of *K. pneumoniae*. However, because of the growing number of publications describing eukaryotic cell invasion by many bacterial pathogens, we investigated the potential invasion ability of *K. pneumoniae*.

This report describes the in vitro ability of a *K. pneumoniae* isolate from a UTI patient to invade human epithelial cells of the bladder and ileocecum in a gentamicin kill-invasion assay. Using a variety of inhibitors that act on eukaryotic cell structures or processes, we investigated the involvement of receptor-mediated endocytosis, endosome acidification, and dependence of microtubules (MT) and microfilaments (MF) on the internalization of *K. pneumoniae*

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MATERIALS AND METHODS

Bacterial strains, cell lines, and culture conditions. *K. pneumoniae* 3091 was isolated from a urine sample of a UTI patient at the Walter Reed Army Medical Center and was kindly provided by R. Almazan. *Salmonella typhi* Ty2 and *E. coli* HB101 were obtained from the strain collection of the Department of Bacterial Immunology, Walter Reed Army Medical Center. Bacteria were kept at −20°C in 50% glycerol in distilled water with 3 mM $MgSO_4$. Bacterial cultures were grown in Luria broth under aeration or on tryptic soy agar at 37°C. After transformation of *K. pneumoniae* 3091 with *gfp* (green fluorescence protein)-expressing plasmid pHO223, 3091/pHO223 was cultivated in the presence of 30 μg of kanamycin per ml to counterselect for loss of the plasmid. Human cell lines were obtained from the American Type Culture Collection (Rockville, Md.) and kept in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide under liquid nitrogen and cultivated at 37°C in an atmosphere of 94% air–6% CO_2. Human embryonic intestinal epithelial INT407 cells were grown in minimal essential medium containing 10% FBS, 2 mM glutamine, and 0.1 mM nonessential amino acids. Cells were passaged at a ratio of 1:10 twice each week. Human epithelial bladder cell lines, T24 and 5637, were cultivated in McCoy's 5A medium with 10% FBS and in RPMI 1640 medium with 10% FBS, respectively. T24 cultures were subcultivated at a ratio of 1:8, and 5637 cultures were subcultivated at a ratio of 1:4 twice each week. HCT-8, a human ileocecal epithelial cell line, was maintained in RPMI 1640 medium supplemented with 1 mM pyruvate, 2 mM glutamine, and 10% FBS. It was passaged at a ratio of 1:5 twice each week. All cell culture media and supplements were obtained from GIBCO/BRL (Gaithersburg, Md.).

Invasion assays. Invasion assays were performed essentially as described earlier (14). In each well of a 24-well cell culture cluster, about 2×10^6 mid-log-phase bacteria (A_{600} = 0.4 to 0.6) were added to confluent monolayers of about 7×10^4 epithelial cells per well (~30 bacteria per epithelial cell). After centrifugation at 200 × *g* for 5 min, invasion was allowed to occur for 2 h at 37°C in an atmosphere of 94% air–6% CO_2. Before a second 2-h incubation (kill) period under the same conditions but with fresh medium containing 100 μg of gentamicin per ml, monolayers were washed once with Earle's balanced salts solution (EBSS). During the kill period, all extracellular bacteria were killed but the viability of internalized bacteria was not affected. Finally, monolayers were washed twice with EBSS and lysed with 0.1% Triton X-100 and the released

intracellular bacteria were enumerated by plate count. Invasion ability was expressed as the percentage of inoculum that survived gentamicin treatment (i.e., recovery). All assays were conducted in duplicate and repeated independently at least three times. The results given are averages of replicate experiments.

Invasion assays in the presence of biochemical inhibitors. Substances that act on eukaryotic cells were added to monolayers 1 h before invasion assays were started in order to study the cell structures and processes involved in internalization. Bacteriostatic antibiotics (chloramphenicol, rifampin, and novobiocin) were added along with bacteria. Biochemical inhibitors were present only during invasion and were diluted out by washing monolayers before the kill period. The concentrations of inhibitors were chosen to give the maximal expected effects without changing the viability of epithelial cells or bacteria for the duration of the assay. The cell viability in monolayers was checked microscopically at the end of each assay and before the final wash steps by adding 10 μl of 0.4% Trypan Blue dye (Hazleton Biologics, Lenexa, Kans.); viable cells excluded the dye. Bacterial viability was determined after 2 h of incubation at 37°C in an atmosphere of 94% air–6% CO_2 in the appropriate cell culture medium with and without inhibitors. Only chloramphenicol inhibited bacterial growth; however, as with all other inhibitors, it did not affect bacterial viability during assays.

Fluorescence staining of MF, MT, and clathrin. To visualize the depolymerization of MF and MT and clathrin accumulation, epithelial cells grown on microscopic slides to confluency were fixed and permeabilized by the method of Osborn and Weber (35). MF were subsequently treated directly with 2.5 μg of fluorescein 5-isothiocyanate (FITC)-labeled phalloidin (Sigma Chemical Co., St. Louis, Mo.) per slide and placed in a cell culture incubator for 1 h. To visualize MT, fixed and permeabilized cells were flooded with diluted (1:50) monoclonal mouse anti-α tubulin serum (Sigma). Cells were incubated for 1 h in a cell culture incubator, washed several times with phosphate-buffered saline, and incubated again for 0.5 h with diluted (1:50) polyclonal goat anti-mouse antibodies which were FITC labeled (Sigma). Clathrin accumulation in fixed and permeabilized epithelial cells was demonstrated by incubating cells with diluted (1:100) monoclonal mouse anti-clathrin (Sigma) serum for 0.5 h. Unbound primary antibodies were removed by extensive washing with phosphate-buffered saline. Incubation for 0.5 h with tetramethyl rhodamine isothiocyanate (TRITC)-labeled secondary anti-mouse antibody (diluted 1:100; Sigma) followed. A cover glass was placed on a drop of Vectra Shield (Vector Laboratories, Inc., Burlingame, Calif.) and sealed with colorless nail polish. The slides were examined on a fluorescence microscope at a final magnification of 650 diameters. *K. pneumoniae* 3091 cells were transformed with plasmid pHO233, expressing *gfp*, and were therefore visible by fluorescence microscopy. In control experiments with only the TRITC-labeled secondary antibody, no clathrin staining was observed. Additionally, bacteria in the absence of epithelial cells were not labeled after treatment for fluorescence staining of clathrin.

Invasion assays in the presence of mannose. Adhesion by type 1 pili is inhibited by mannose. To assess the involvement of type 1 pili in internalization, bacteria grown in Luria broth with 1, 10, or 100 μM or 1 mM mannose to mid-log phase (A_{600} = 0.4 to 0.6) were preincubated with epithelial cells in the presence of mannose at the same concentration as that in the bacterial culture for 1 h before the start of invasion assays.

Assay to monitor intracellular replication and persistence. Invasion assays were performed as described above to determine potential bacterial intracellular replication and persistence in epithelial cells, with the following alterations. The invasion period was reduced to 1 h. After this period, gentamicin was added at 100 μg/ml for 2 h and then replaced with fresh medium containing either 100 or 10 μg of gentamicin per ml. Cells were then reincubated until lysed at various times (2 to 96 h).

Transmission electron microscopy. Invasion assays were performed as described above, except that 10-fold-more (7×10^6) cells of *K. pneumoniae* 3091 or *E. coli* HB101 were added to epithelial cells and that monolayers were not treated with gentamicin. Infected cells were washed once with EBSS and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. The material was washed in cacodylate buffer and postfixed with 1% OsO_4 in the same buffer for 2 h at room temperature. The postfixed material was washed in the same buffer, dehydrated in a graded series of ethanol (30 to 100% ethanol) and propylene oxide, and embedded in Epon 812. Ultrathin sections were made on a Leica (Deerfield, Ill.) Ultracut S ultramicrotome. Sections were stained with uranyl acetate and lead citrate by the method of Reynolds (39) and examined under a Philips (Mahway, N.J.) 400 transmission electron microscope at an accelerating voltage of 80 kV.

Observation of intracellular bacteria by light microscopy after acridine orange staining. Monolayers of T24, 5637, and HCT-8 cells were grown overnight on microscopic slides to confluency, infected with mid-log-phase bacteria at a multiplicity of infection (MOI) of ~10, and incubated for 2 h under cell culture conditions. After this invasion period, monolayers were washed twice with EBSS, stained with 0.01% acridine orange in EBSS, and counterstained with 0.05% crystal violet in 0.15 N NaCl for 45 s each time by the method of Miliotis (27). On slides viewed under a fluorescence microscope, intracellular bacteria appeared green. Although extracellular bacteria were not visible because of quenching by crystal violet (27), they were readily observed by phase-contrast microscopy.

TABLE 1. Comparison of invasion efficiencies of *K. pneumoniae* 3091, invasive *S. typhi* Ty2, and noninvasive *E. coli* HB101 for different human epithelial cell lines

Cell line	Human origin	% Recovery[a]		
		3091	Ty2	HB101
T24	Bladder	5.1 ± 2.7	6.3 ± 1.2	0.02 ± 0.01
5637	Bladder	0.6 ± 0.3	4.0 ± 1.6	0.04 ± 0.01
HCT-8	Ileocecum	2.7 ± 0.5	8.4 ± 1.8	0.07 ± 0.05
INT407	Intestine	0.02 ± 0.008	10.2 ± 8.5	0.02 ± 0.01

[a] Percentage of inoculum that survived gentamicin treatment. Data are the means ± standard deviations of at least four independent experiments carried out in duplicate.

RESULTS

Invasion of human epithelial cell lines by *K. pneumoniae*. Four human epithelial cell lines (two from the human bladder and two of intestinal origin) were used to determine the invasion ability of *K. pneumoniae* 3091. For comparison, the uptake efficiencies of invasive *S. typhi* Ty2 and noninvasive *E. coli* HB101 were assessed in concurrent experiments. *K. pneumoniae* 3091 was highly invasive for T24 bladder and HCT-8 ileocecal cells, with recoveries (i.e., percentages of inoculum that survived gentamicin treatment) of 5.1% ± 2.7% and 2.7% ± 0.5%, respectively (Table 1). The intracellular 3091 number was comparable to that of Ty2 in T24 cells (6.3% ± 1.2%) and lower than that of Ty2 in HCT-8 cells (8.4% ± 1.8%) (Table 1). *K. pneumoniae* recovery after invasion of 5637 bladder cells was low (0.6% ± 0.3%) but still ~15-fold above that of the negative control, HB101 (Table 1). The human embryonic intestinal cell line INT407 was not invaded by *K. pneumoniae* above the level of HB101, although Ty2 entered this cell line very efficiently (recovery, 10.2% ± 8.5%) (Table 1). Therefore, the *K. pneumoniae* internalization mechanism was further characterized only with the three invasion-positive cell lines.

Effects of different MOIs on internalization efficiency. Besides the MOI of 10 used in standard invasion assays, lower and higher MOIs were used. Lowering the MOI below 10 had the effect of increasing the percentage of recovery, whereas raising the MOI had the effect of reducing the percentage of recovery (Fig. 1). In contrast, lowering the MOI decreased the number of intracellular bacteria per epithelial cell, whereas raising the MOI increased the number of intracellular bacteria per cell (Fig. 1). These effects were found with all three cell lines (T24, 5637, and HCT-8). At an MOI of about 200, four bacteria were internalized per epithelial T24 cell and all epithelial cells that constituted the monolayer were infected by *K. pneumoniae* 3091 during a 2-h invasion period (Fig. 1A). At the same MOI, only 70% of HCT-8 cells were infected (i.e., 0.7 intracellular bacterium per HCT-8 cell) (Fig. 1B). Higher MOIs caused rapid epithelial cell death and disintegration of the monolayer (data not shown).

Bacterial intracellular replication and persistence. Invasion assays were performed as described above to determine the extent of intracellular replication and persistence of *K. pneumoniae* 3091 in bladder cells. For that purpose, two assays were conducted in parallel. For both assays, the initial 1-h internalization period and 2-h gentamicin kill period with a gentamicin concentration of 100 μg/ml were performed as described above. However, during the remaining portion of the experiment, the medium was removed in both assays after the initial 2-h gentamicin kill period and replaced with fresh medium containing either 10 or 100 μg of gentamicin per ml. Infected monolayers were reincubated and then lysed at various times

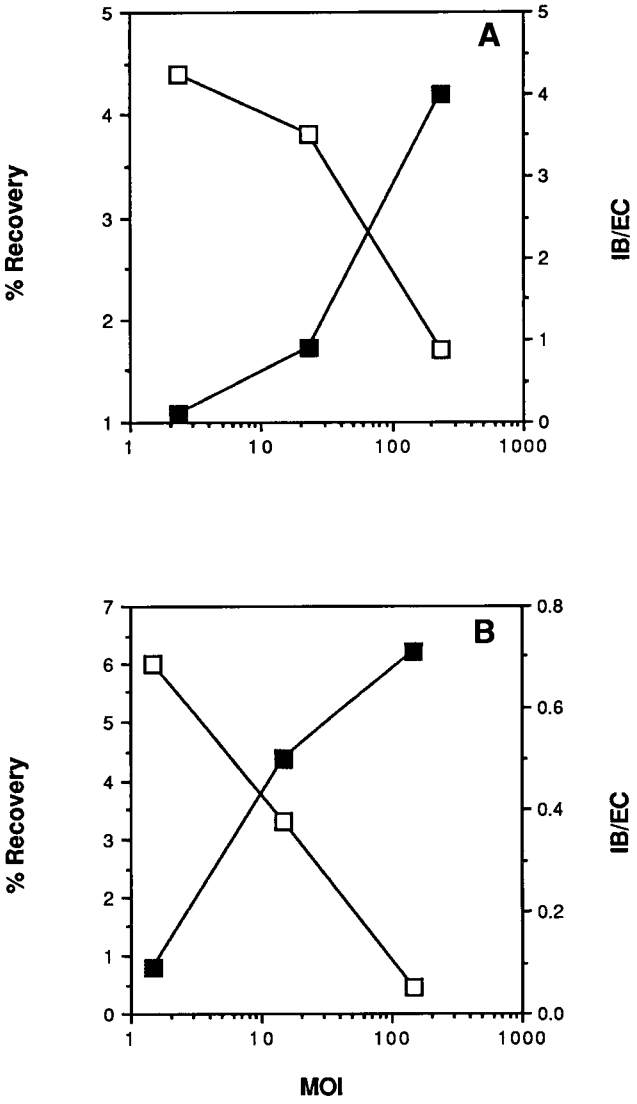

FIG. 1. Effects of different MOIs on the percentage of inoculum that survived gentamicin treatment (% Recovery; open squares) and number of intracellular bacteria per epithelial cell (IB/EC; solid squares). Confluent monolayers of T24 bladder epithelial cells (A) and HCT-8 ileocecal epithelial cells (B) were infected with *K. pneumoniae* 3091 at MOIs of about 2 to 200. Similar results were obtained with 5637 bladder epithelial cells. Invasion was allowed to occur for 2 h.

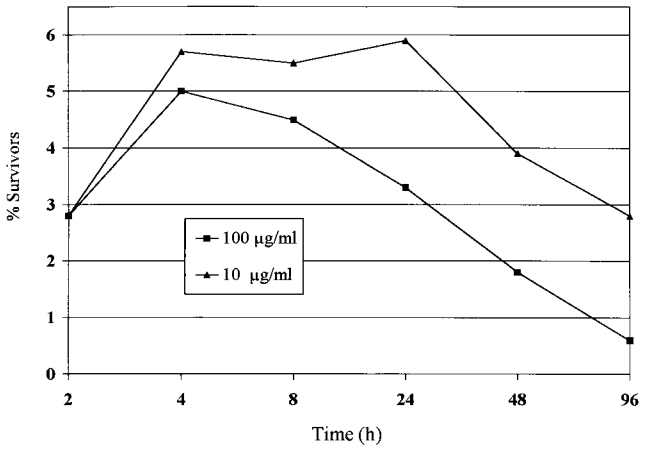

FIG. 2. Intracellular survival of *K. pneumoniae* 3091 in T24 bladder epithelial cells grown in wells of a 24-well tissue culture plate over 4 days. Monolayers were infected with strain 3091 and incubated for 1 h in a cell culture incubator. After invasion, the original medium was replaced with fresh medium containing gentamicin (100 μg/ml). Two hours later, the medium was changed again. In half of the wells, the medium was replaced with medium containing 100 μg of gentamicin per ml (final concentration). The medium was replaced in the other half of the wells with medium containing 10 μg of gentamicin per ml. Infected monolayers were lysed at different times, and the number of intracellular bacteria was compared with that at 2 h after gentamicin was added (i.e., 100% recovery).

(2 to 96 h). The results showed that the number of intracellular *K. pneumoniae* cells recovered in the presence of 10 μg of gentamicin per ml doubled during the first 4 h and was unchanged during the subsequent 20 h. Thereafter (the following 3 days), the number of intracellular bacteria recovered declined steadily, approaching the size of the original inoculum by 96 h (Fig. 2). In contrast, in the presence of 100 μg of gentamicin per ml, the number of intracellular *K. pneumoniae* cells recovered again nearly doubled by 4 h but steadily decreased thereafter, reaching approximately 20% of the size of the original inoculum by 96 h (Fig. 2).

Invasion efficiency in the presence of mannose. The adherence of *K. pneumoniae* to human (36) and rat (16) bladder cells is mediated by type 1 fimbriae (17) and inhibited by mannose (33). Although the *K. pneumoniae* UTI isolate, 3091, from our glycerol stock seemed to be nonpiliated, bacteria expressed pili after daily transfer for 1 week in tryptic soy broth with 5 g of glucose per liter as previously described (16). The presence of pili was determined by hemagglutination of guinea pig erythrocytes, which occurred only with tryptic soy broth- and glucose-passaged cultures of 3091. This culture also produced a pellicle, which is characteristic of piliated enteric bacteria (11). The invasion of T24, 5637, and HCT-8 cells by nonpiliated and piliated forms of 3091 was equally efficient and independent of the presence or absence of mannose (1 to 1,000 μM) in the growth media of both bacteria and cells (data not shown).

Bacterial de novo protein synthesis and invasion. Because it is known that bacterial de novo protein synthesis is necessary for efficient invasion by *Campylobacter jejuni*, *Citrobacter freundii*, and *S. typhi* (32) and *S. cholerae-suis* (20) but not for invasion by *Yersinia enterocolitica* (19), the dependency of *K. pneumoniae* invasion on de novo protein synthesis was investigated. At the start of the invasion assay, chloramphenicol (100 μg/ml) was added along with bacteria to wells with the epithelial cell monolayer, as described above. The invasion ability of strain 3091 was reduced in the presence of chloramphenicol by 98% in T24 cells (Table 2), by >99% in 5637 cells, and by >99% in HCT-8 cells. At the concentration used, chloramphenicol inhibited the growth of 3091 in the cell culture medium but did not reduce bacterial viability during 2 h of incubation at 37°C in a cell culture incubator (Table 2). The invasion ability of strain 3091 was also reduced by the addition of rifampin or novobiocin together with bacteria to epithelial cells. However, the effect of inhibition on bacterial mRNA and DNA synthesis, which reduced internalization efficiency, was not as dramatic as the inhibition of bacterial protein synthesis (Table 2). The reduced uptake of strain 3091 was not due to reduced viability in the presence of any of these bacteriostatic antibiotics. Although *K. pneumoniae* growth was inhibited in the presence of chloramphenicol and reduced in the presence of rifampin or novobiocin, viability was not reduced (Table 2).

Inhibition of receptor-mediated endocytosis and its effect on bacterial uptake. Because the uptake into eukaryotic cells of certain viruses, *Chlamydia* spp. (38, 44, 50), and *Campylobacter*

TABLE 2. Effects of inhibition of bacterial protein, mRNA, and DNA synthesis on growth and internalization efficiencies into T24 cells

Bacteriostatic antibiotic[a]	No. of survivors[b]		% Relative invasiveness[c]	
	3091	Ty2	3091	Ty2
None	222×10^5	744×10^5	100	100
Chloramphenicol	4.8×10^5	21.4×10^5	0.2 ± 0.1	0.4 ± 0.3
Rifampin	14.4×10^5	22.5×10^5	21 ± 13	0.5 ± 0.2
Novobiocin	16.9×10^5	34.9×10^5	5 ± 3	35 ± 12

[a] Together with bacteria, 100 μg of chloramphenicol per ml, 100 μg of rifampin per ml, 50 μg of novobiocin per ml, or no antibiotic was added.

[b] To determine the effects of antibiotics on viability, bacteria were incubated in cell culture medium for T24 cells with and without the indicated antibiotic for 2 h under cell culture conditions. The size of the inoculum was about the same as at the beginning of invasion assays (i.e., 5.37×10^5 for 3091 and 23.25×10^5 for Ty2). After incubation, the titer of bacteria in medium with or without antibiotic was determined. The data given are for a typical experiment. The standard deviation never exceeded 17% of the mean.

[c] Invasion assays were performed as described in Materials and Methods. Data were plotted so that relative invasiveness in the absence of any antibiotic equalled 100%.

jejuni and *Citrobacter freundii* (32) may occur via coated pits or by receptor-mediated endocytosis, we tried to demonstrate the involvement of coated pits and receptor-mediated endocytosis as part of the internalization process of *K. pneumoniae*. The formation of coated pits and uptake via coated pits can be inhibited by treating epithelial cells with g-strophanthin (gS) (13, 24) or monodansylcadaverine (MD) (7). In contrast to the work of Davies et al. (7), other studies have implied that MD inhibits receptor-mediated endocytosis (48). The relative invasion of HCT-8 cells by *K. pneumoniae* 3091 was reduced by gS and MD to 17 and 19%, respectively; however, the entry of 3091 into T24 cells was inhibited only by MD (31% relative invasion), not by gS, whereas internalization into 5637 cells was only marginally reduced by these inhibitors (Fig. 3). In general, the invasion ability of *S. typhi* Ty2 was not affected by gS or MD (Fig. 3). In control experiments, the incorporation of [^{35}S]methionine into unprotected (no MD) and protected (250 μM MD) epithelial cells in the presence and absence of 10 nM diphtheria toxin was assessed by the method of Moya et al. (28). The incorporation rate of [^{35}S]methionine was only 3% for unprotected cells versus 100% for protected cells in the presence of diphtheria toxin, compared with that seen in the presence of MD (i.e., 100% incorporation of [^{35}S]methionine). The involvement of clathrin in *K. pneumoniae* internalization was demonstrated by indirect immunofluorescence. Colocalization of clathrin with many but not all epithelial cell-associated *K. pneumoniae* cells was clearly visible after staining with a monoclonal mouse antibody directed to clathrin and a secondary antibody labeled with TRITC (Fig. 4). Bacteria were visualized after transformation with *gfp* (green fluorescence protein) gene-expressing plasmid pHO233 (Fig. 4). The *gfp* transformant was found to be as invasive as its parental type strain (data not shown).

Effects of inhibition of endosome acidification on bacterial internalization and intracellular bacterial survival. The acidification of endosomes can be inhibited by amines, such as methylamine, chloroquine, and NH_4Cl (8), and by monensin, an ionophore that exchanges protons for sodium ions. Final concentrations of NH_4Cl to 20 mM had no marked effect on the relative invasion of 3091 into T24 and 5637 cells (Fig. 3). On the other hand, chloroquine (40 μg/ml) reduced the numbers of viable intracellular *Klebsiella* and *Salmonella* cells (data not shown). This was also the case for a variety of studied

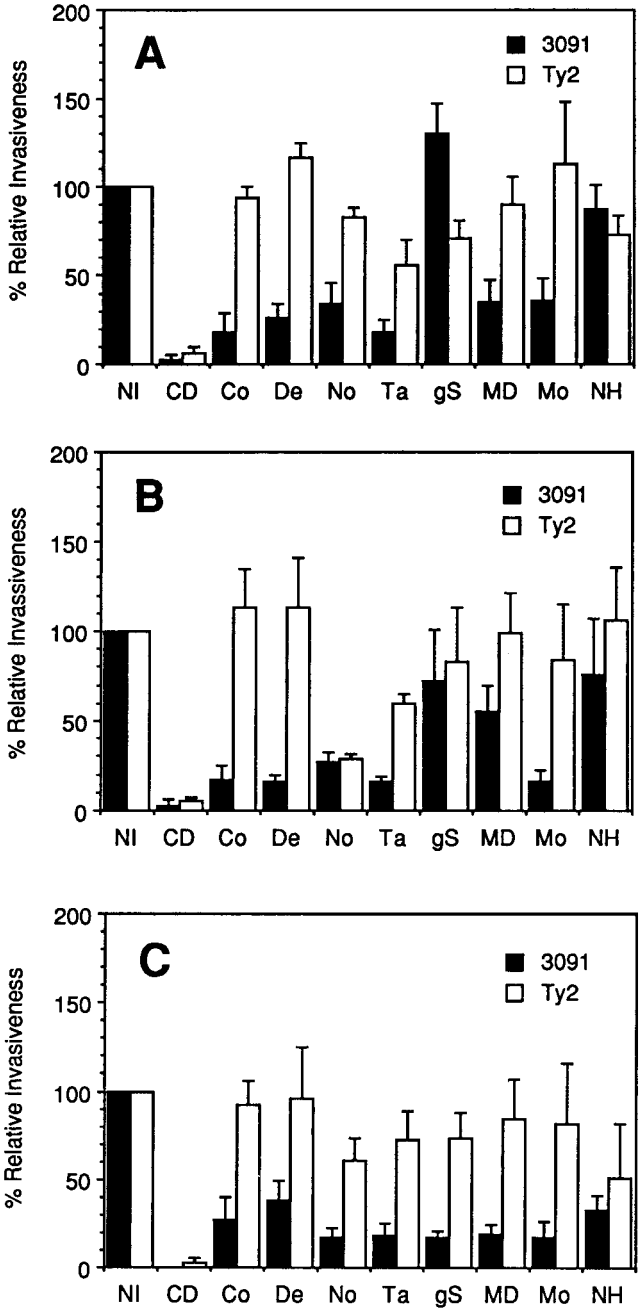

FIG. 3. Effects of different inhibitors on the internalization of *K. pneumoniae* 3091 and *S. typhi* Ty2 into human epithelial cell lines of the bladder (T24 [A] and 5637 [B]) and ileocecum (HCT-8 [C]). The inhibitors cytochalasin D (CD; 2 μM), colchicine (Co; 10 μM), demecolcine (De; 1 μM), nocodazole (No; 20 μM), taxol (Ta; 40 μM), gS (250 μM), MD (250 μM), monensin (Mo; 40 μM), and ammonium chloride (NH; 20 mM) were added 1 h before the addition of bacteria and were present until gentamicin was added (see Materials and Methods). The number of intracellular bacteria after invasion in the presence of an inhibitor was divided by the number of intracellular bacteria in the absence of inhibitors and multiplied by 100 to calculate the percentage of relative invasiveness. Invasion efficiency in the absence of inhibitors (NI) was defined as 100% relative invasiveness. Each column represents the average of at least three results in duplicate. Standard deviations are given as error bars.

facultative intracellular bacteria and may be caused by the endosomal accumulation of chloroquine to bacteriocidal concentrations (18). The only exception is *Shigella flexneri*, which escapes from the endosome (19). Monensin (40 μM) reduced

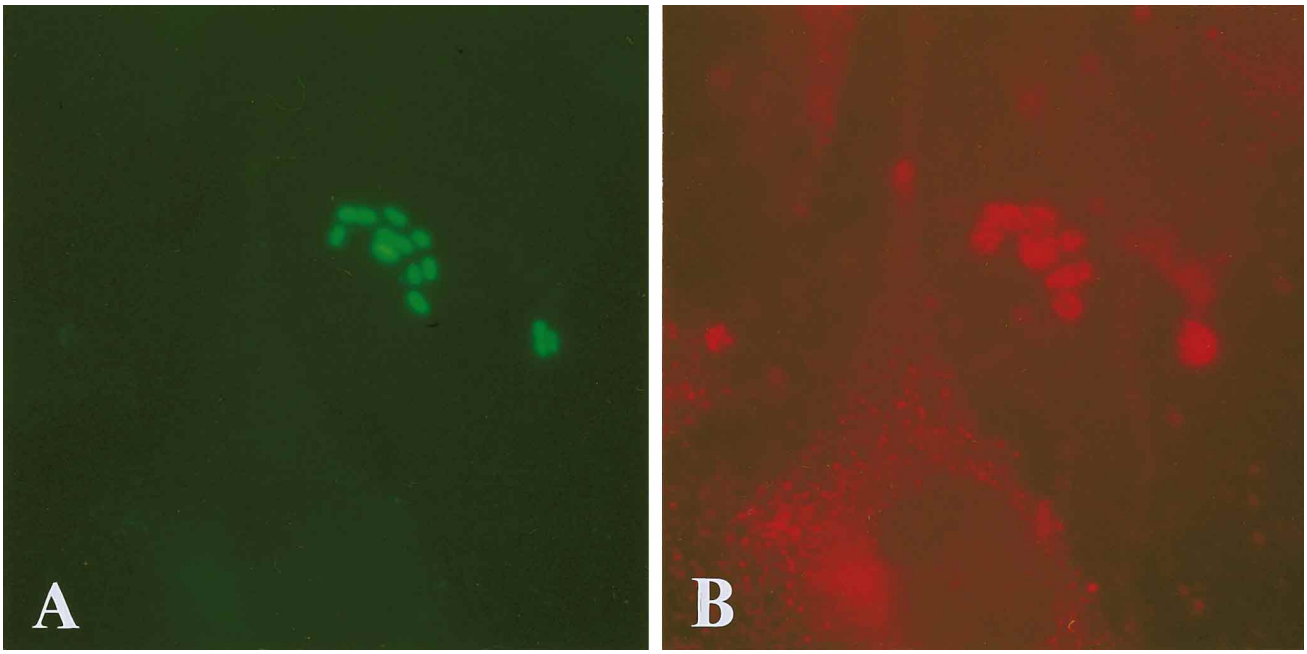

FIG. 4. Fluorescence microscopy of *gfp*-expressing *K. pneumoniae* (A) and indirect immunofluorescence for visualization of clathrin (B) in T24 bladder cells. Monolayers of T24 cells cultivated on glass coverslips were infected with *K. pneumoniae* 3091/pHO223 for 2 h and then processed for fluorescence microscopy as described in Materials and Methods.

the relative invasion of *K. pneumoniae* 3091 but not *S. typhi* Ty2 into bladder and ileocecal cell lines (Fig. 3). To determine whether the monensin effect was due to impaired uptake or reduced intracellular survival, the invasion efficiency of 3091 into untreated cells was compared with that in cell lines treated with monensin 1 h before the start of the invasion assay and maintained during the invasion period and with the survival within monolayers to which monensin was added along with gentamicin (i.e., monensin was present only after the invasion period). The results differed somewhat from cell line to cell line. The relative invasion of 3091 into T24 cells was reduced by 63% when monensin was present during the invasion period (Fig. 3A) and by 44% when monensin was present during the kill period. Monensin reduced the relative invasion by 84 and 83% during the uptake of 3091 into 5637 and HCT-8 cells, respectively (Fig. 3B and C). When monensin was present during the kill period, it reduced in 5637 cells the number of viable intracellular *K. pneumoniae* cells by only 37% and slightly increased (38%) the number in HCT-8 cells. In contrast, the invasion ability of Ty2 was not affected by monensin (Fig. 3).

Invasion of epithelial cells treated with cytochalasin D. Cytochalasin D, an agent more specific and potent than is cytochalasin B (4) for depolymerizing MF, was used to study the involvement of MF during the entry of 3091 into human epithelial cells. *S. typhi* Ty2 served as a positive control because of its strict dependency on MF for efficient invasion of INT407, T24, and 5637 cells (14, 32). Cytochalasin D (2 μM) reduced the relative invasion of Ty2 and *K. pneumoniae* 3091 into all three cell lines studied. The average reduction in 3091 invasion ability was >93% (Fig. 3). MF depolymerization was visualized by fluorescence staining of F-actin with FITC-labeled phalloidin. Defined MF were observed by fluorescence microscopy only in untreated epithelial cells, not in cells treated for 1 h with 2 μM cytochalasin D (data not shown).

Effects of depolymerization of MT on invasion by *K. pneumoniae*. Besides MF, MT are major component of the cytoskeleton. They can be depolymerized by three inhibitors, colchicine, demecolcine, and nocodazole. Depolymerization of MT was verified by indirect immunofluorescence labeling of MT with monoclonal mouse anti-α tubulin and FITC-labeled goat anti-mouse antibodies. Untreated epithelial cells showed well-defined MT. No MT were observed in epithelial cells treated for 1 h with 10 μM colchicine, 1 μM demecolcine, or 20 μM nocodazole (data not shown). MT depolymerization by colchicine and demecolcine did not affect or only slightly reduced Ty2 invasion of T24 and 5637 cells (Fig. 3A and B) (32) and HCT-8 cells (Fig. 3C). In contrast, both substances, as well as nocodazole, markedly inhibited 3091 entry into all three cell lines (Fig. 3). Nocodazole reduced the relative invasion of Ty2 into 5637 and, to some extent, HCT-8 cells (Fig. 3B and C) (32).

Effects of MT stabilization on invasion. To a great extent, depolymerization of MT can be prevented by taxol (3, 41). The stabilization of MT in the presence of 50 μM taxol had only a slight effect on Ty2 invasion of the cell lines used in this study (Fig. 3). However, the internalization of *K. pneumoniae* 3091 into bladder and ileocecal cells was inhibited by taxol (>80%) to the same or an even greater extent than it was by MT depolymerization (Fig. 3). The stabilizing effect of taxol on MT was shown in control experiments by indirect immunofluorescence labeling of MT in cells untreated or treated with taxol (40 μM for 1 h). Depolymerization of MT after incubation with the MT depolymerizer demecolcine (1 μM for 1 h) occurred only in cells not pretreated with taxol (data not shown).

Electron microscopic examination of epithelial cell monolayers infected with *K. pneumoniae*. To verify invasion ability by a direct method rather than indirect determination via invasion assays (i.e., protection from the bactericidal action of gentamicin), infected monolayers were fixed at different times after

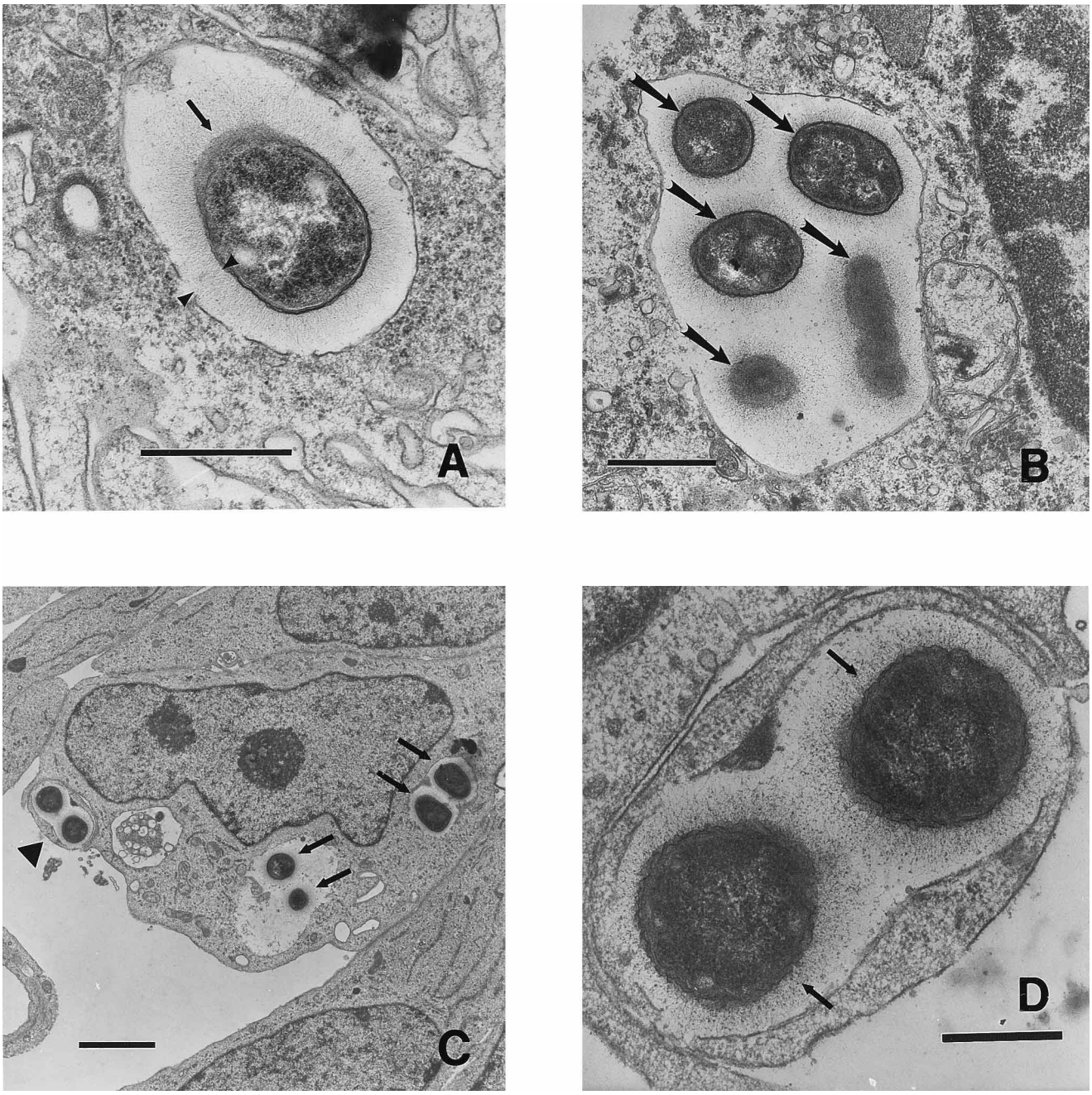

FIG. 5. Transmission electron photomicrographs of T24 cells infected with *K. pneumoniae* 3091. Infected monolayers were processed for electron microscopic examination as described in Materials and Methods. Intracellular bacteria (arrows) were observed in endosomes as individual organisms (A) or in groups (B), revealing an electron-dense fibrous surface structure (area between small arrowheads surrounding bacterium in panel A). (C) Some epithelial cells contained numerous endosomes, some of which harbored bacteria. (D) Area in panel C indicated by large arrowhead at a higher magnification, with two intracellular bacteria in what seems to be a cell protrusion being internalized by a second, neighboring cell. Bars: 1 (A and B), 2 (C), and 0.5 (D) μm.

the addition of bacteria and prepared for electron microscopic examination. At 1 h and later times after infection of monolayers, *K. pneumoniae* cells were seen intracellularly, always in an endosome (Fig. 5A). An electron-dense fibrous surface structure was observed on bacteria adhering to epithelial cells and on bacteria within endosomes. This finding suggests that the surface structure did not interfere with the internalization process. The finding of endosomes containing numerous bacterial cells (some with septa) in bladder epithelial cells suggests that either intraendosomal bacteria replication or uptake of whole microcolonies occurred by 3 h postinfection (Fig. 5B). At 3 h, many bladder cells possessed multiple bacteria-filled endosomes (Fig. 5C). Sometimes it appeared as if the bacteria in endosomes in one epithelial cell were being internalized by neighboring cells (Fig. 5D). In contrast, internalized *E. coli* HB101 cells within epithelial cells were not observed.

Light microscopic examination of infected monolayers stained with acridine orange. Confluent monolayers on micro-

scopic slides were infected with strain 3091 as described above. After the 2-h invasion period, infected monolayers were stained with acridine orange and counterstained with crystal violet so that intracellular bacteria could be visualized by fluorescence microscopy. Intracellular *K. pneumoniae* cells appeared as green-fluorescing bacteria but were not visible by phase-contrast microscopy (not shown). Bacteria visible by phase-contrast microscopy did not fluoresce under blue light illumination (not shown). Green-fluorescing bacteria were also observed with monolayers infected with *S. typhi* Ty2. In contrast, epithelial cells infected with *E. coli* HB101 only rarely revealed fluorescing bacteria (data not shown). The absence of red-fluorescing bacteria indicated that all intracellular bacteria were viable, since nonviable intracellular bacteria would have stained red (21).

DISCUSSION

For the first time, we describe here the ability of *K. pneumoniae* to invade cultured human epithelial cells in vitro. Intracellular *K. pneumoniae* locations were determined by three techniques, invasion assays with gentamicin, electron microscopy, and fluorescence microscopy. Although the relevance of in vitro invasion to *K. pneumoniae* pathogenesis is not known, hiding in epithelial cells and persisting there for at least several days would give *K. pneumoniae* the opportunity to escape from the host's humoral immune mechanisms and to establish an acute infection.

Adherence is thought to be a prerequisite for bacterial triggering of internalization, but type 1 pili seemed not to be essential for invasion by 3091 (i.e., mannose did not inhibit invasion ability). This finding does not exclude the possibility that 3091 adheres through other factors. Electron microscopic examinations revealed an electron-dense fibrous external layer (Fig. 5) radiating from the cell surface which may be a mannose-resistant adherence factor, such as MR/K, type 3, or type 6 fimbriae, and may be involved in adherence (12, 34, 37). Alternatively, these surface structures may represent capsular material.

De novo protein synthesis is necessary for efficient invasion by *K. pneumoniae* 3091, as seen for a variety of bacteria, and may reflect the synthesis of bacterial ligands with short half-lives (e.g., by protein degradation or inactivation of the protein ligand) necessary for uptake. Once intracellular, the number of *K. pneumoniae* cells increased only during the first 4 h after gentamicin addition by one round of replication, but *K. pneumoniae* cells survived intracellularly for at least 4 days (Fig. 2).

The inhibition of endosome acidification by monensin reduced the relative invasion ability of 3091 by interfering mainly with uptake and far less with the ability to survive intracellularly. The number of intracellular *K. pneumoniae* cells was somewhat reduced in the presence of monensin during the kill period in T24 and 5637 cells. This is very unlikely due to a direct bacteriocidal effect of monensin, since monensin did not affect the growth of *K. pneumoniae* in control experiments under assay conditions without epithelial cells. Monensin might have caused some leakiness of these epithelial cells for gentamicin or might have affected to some extent the expression of bacterial genes necessary for intracellular survival in T24 and 5637 cells. However, internalization was shown to be much more or similarly affected by monensin in all three cell lines. The trapping of receptors used by 3091 to trigger internalization in unacidified endosomes (i.e., acidification releases endosome-bound receptors and allows for recycling to cell surface) may be the cause of the monensin effect. Such trapping has been shown for a number of receptors, such as those of low-density lipoprotein (2) and asialoglycoprotein (42). Other bacteria, such as *Brucella abortus* (9), *Citrobacter freundii* (32), and *Helicobacter pylori* (15), are thought to rely on endosome acidification for successful entry. However, the reported invasion efficiencies of *B. abortus* and *H. pylori* were extremely low (i.e., in the range of noninvasive *E. coli*) compared with those of *Citrobacter freundii* and *K. pneumoniae*. Therefore, we question the significance of invasion by *B. abortus* and *H. pylori* and their dependency on endosome acidification for invasion.

All well-characterized entry systems triggered by bacteria depend heavily on MF, except the internalization mechanisms of *Campylobacter jejuni* into INT407 cells and *Citrobacter freundii* into T24 cells (32). These two species are dependent on MT for efficient uptake in the aforementioned cell lines. However, as observed for the internalization of *K. pneumoniae* 3091, the invasion ability of *Citrobacter freundii* for other cell lines is not only MT dependent but also severely reduced by cytochalasin D, indicating the essential role of MF for efficient entry. Unlike *Citrobacter freundii*, however, invasion by *K. pneumoniae* was also inhibited by the stabilization of MT by taxol. The involvement of MT in the uptake mechanisms of *Chlamydia trachomatis* (49), *Haemophilus influenzae* (46), *H. pylori* (15), *Neisseria gonorrhoeae* (40), and enteropathogenic *E. coli* (10) has also been reported. However, only the invasion efficiencies of *Chlamydia trachomatis* and enteropathogenic *E. coli* equalled those of *Citrobacter freundii* and *K. pneumoniae*. Although colchicine and demecolcine did not reduce Ty2 entry, nocodazole inhibited Ty2 uptake into 5637 cells and to a lesser extent into HCT-8 cells. This might be due to the unknown secondary effects of nocodazole. Interestingly, if bacterial uptake involves both MF and MT, the inhibitory effect of MF depolymerization on bacterial internalization is always much more pronounced than that of MT depolymerization. This was observed not only for *K. pneumoniae* but also for internalization of enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, and *E. coli* associated with newborn meningitis (10, 25, 30). However, the inhibition of engulfment by MT depolymerization can be as severe for solely MT-dependent internalization as that by MF depolymerization is for solely MF-dependent uptake. This was demonstrated for the entry of *Campylobacter jejuni* 81-176 into INT407 cells (32). Obviously, in uptake pathways that involve both MT and MF cytoskeleton elements, internalization still occurs at a substantial level even after the application of MT depolymerizers. This may indicate that two independent pathways of internalization are used by *K. pneumoniae* or that MF can compensate to some extent for MT function in the uptake process. Another interpretation of the data is that MT are not 100% depolymerized in epithelial cells by treatment with colchicine, demecolcine, or nocodazole. Thus, the remaining MT are sufficient to support substantial internalization. However, no remaining MT were detectable by indirect immunofluorescence of colchicine-, demecolcine-, or nocodazole-treated epithelial cells.

K. pneumoniae 3091 represents another bacterial species, in addition to *B. abortus*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Campylobacter jejuni*, *Citrobacter freundii*, *Proteus mirabilis*, and *H. pylori*, for which entry into some cell lines is inhibited by interference with receptor-mediated endocytosis (9, 15, 23, 31, 32, 44). It remains to be seen whether it is coincidental that the internalization of these species is also inhibited by depolymerization of MT or whether entry via coated pits is the first step followed by further inward transport of the invading bacterium via MT as a second step of a coherent invasion pathway. Reduced uptake into MD-treated cells might additionally or solely be due to the inhibitory effect of MD on transglutaminases essential for receptor recycling, thus

leading to depletion of surface-exposed receptors necessary for efficient uptake of *K. pneumoniae* (48). Nevertheless, reduced internalization of *K. pneumoniae* into MD-treated cells suggests that receptors recycled through endosomes are involved in the uptake of *K. pneumoniae* into human epithelial cells.

Taken together, these results suggest that *K. pneumoniae* 3091 triggers an uptake mechanism(s) into human epithelial cell lines that depends on MF, MT, and a receptor(s) which is recycled through endosomes and reflects receptor-mediated endocytosis most likely into HCT-8 and T24 cells. However, in contrast to the MT-dependent internalization mechanisms of *Campylobacter jejuni* and *Citrobacter freundii*, the *K. pneumoniae* mechanism is sensitive to the stabilization of MT by taxol. Immunocytochemistry studies are under way to provide further insight into the molecular mechanism of *K. pneumoniae* internalization. Furthermore, molecular cloning of the *K. pneumoniae* genes necessary for efficient invasion will establish the genetic basis of the uptake mechanism described here.

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