

Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*

(coated-pits/cytoskeleton/endosome acidification/microfilaments/protein synthesis)

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ABSTRACT Bacterial invasion of six different human epithelial cell lines showed that some strains of the intestinal pathogen *Campylobacter jejuni* invaded intestinal cell lines at a level 10^2 – 10^4 times higher than reported previously for other *Campylobacter* strains. Separately, urinary tract isolates of *Citrobacter freundii* triggered a high-efficiency invasion of bladder cells. Use of multiple inhibitors with known effects on eukaryotic cell structures/processes allowed us to define in these genetically distinct bacterial genera unusual bacterial invasion mechanisms that uniquely require microtubules but not microfilaments. *Campylobacter jejuni* strain 81-176 uptake into 407 intestinal cells and *Citrobacter* entry into T24 bladder cells was blocked by microtubule depolymerization and inhibitors of coated-pit formation but not by microfilament depolymerization. Inhibitors of endosome acidification had no significant impact on intracellular survival of *Campylobacter jejuni* or *Citrobacter freundii*, but monensin markedly reduced *Citrobacter* uptake. Epithelial cell invasion by both of these bacterial genera was dependent upon *de novo* bacterial protein synthesis but not upon *de novo* eukaryotic cell protein synthesis. In contrast to the T24 cell line-specific, strict microtubule-dependent uptake, *Citrobacter* entry into other cell lines was inhibited by both microtubule- and microfilament-depolymerization, suggesting that these bacteria encode two separate pathways for uptake (*i*, microtubule-dependent; *ii*, microfilament-dependent) that are cell line-specific and are recognized perhaps depending on the presence and abundance of appropriate eukaryotic receptors.

The *Campylobacter* spp. *C. jejuni* and *C. coli* are among the most common causes of human diarrheal diseases (1, 2). The pathophysiology of diarrheal disease(s) caused by *Campylobacter* is poorly understood, although volunteer studies have revealed that oral doses of $\leq 10^3$ *Campylobacter jejuni* can cause illness (3). Clinical symptoms range from a protracted watery diarrhea to bloody diarrhea with fever, abdominal cramps, and the presence of fecal leukocytes. Reports of *Campylobacter* bacteremia during episodes of gastroenteritis (4) and the results of intestinal biopsies of patients or infected primates (5) suggest that at least some *Campylobacter* spp. cause an invasive disease of the intestine. The ability of *Campylobacter jejuni* to invade enterocytes has been confirmed in experimental animal models with orally infected infant chickens (6), mice (7), and hamsters (8). In addition, a microfilament (MF)-dependent, extremely low-level invasion of *Campylobacter jejuni* into cultured human epithelial cells has been reported previously (9–11).

Although *Citrobacter freundii* is often considered an enterobacterial commensal of the human intestinal flora, it has been associated with diarrhea in children (12) and has been reported to cause invasive infections such as neonatal meningitis with a high mortality (13), isolated cases of pyomyo-

itis (14), and bacteremia presenting as typhoid fever (15). Additionally, *Citrobacter freundii* is recognized as an occasional causative agent of urinary tract infections (UTI). Still, there is little known about its pathogenic mechanisms, including the potential involvement of invasive ability, besides the production in some strains of a heat-stable enterotoxin identical with ST1a of *Escherichia coli* (12).

In this study, we have analyzed pathogenic isolates of these two distinct genera for their ability to invade several different lines of cultured human epithelial cells, primarily of the intestine or bladder. We define below the presence of relatively high-level epithelial cell invasion systems in both genera and have further characterized the uptake mechanisms through the use of biochemical inhibitors of prokaryotic or eukaryotic processes/structures and by electron microscopy. In contrast to the well-characterized epithelial cell invasion systems triggered by enteroinvasive *E. coli*, *Legionella*, *Listeria*, *Salmonella*, *Shigella*, and *Yersinia* spp., which are all strictly MF-dependent, *Campylobacter jejuni* and *Citrobacter freundii* initiate distinctive microtubule (MT)-dependent, endocytic processes, which result in their uptake into endosomal vacuoles.

MATERIALS AND METHODS

Bacterial Strains, Cell Lines, Media, and Culture Conditions. *E. coli* HB101 and *Salmonella typhi* Ty2 (Walter Reed Army Institute of Research strain collection) served as negative and positive invasion controls, respectively. *Citrobacter freundii* strains 3009 and 3056, isolated recently from UTI patients at Walter Reed Hospital, were provided by R. Almazan. These bacteria were routinely cultivated in L broth or on TSA agar (Difco) at 37°C. *Campylobacter jejuni* strain 81-176 was obtained from the stool of a volunteer with colitis following a human feeding study (3), and human disease isolate A3249 was provided by R. E. Black (Johns Hopkins University). *Campylobacter jejuni* VC84 and 3535 are human disease isolates obtained from T. J. Trust (University of Victoria, British Columbia), and D. N. Taylor (Walter Reed Army Institute of Research). *Campylobacter* strains were grown in Mueller–Hinton biphasic medium, on Mueller–Hinton agar (Difco) or in minimal essential medium with 10% (vol/vol) fetal calf serum at 37°C in a 6% CO₂/94% air atmosphere. The human cell lines of the embryonic intestine (INT407), the bladder (T24 and S637), the cervix (HeLa), the ileocecum (HCT-8), and the kidney (A-498), obtained from American Type Culture Collection, were maintained in liquid nitrogen and cultivated in the media and split as suggested by American Type Culture Collection.

Invasion Assay. These assays were performed essentially as described by Elsinghorst *et al.* (16). To a confluent monolayer of about 7×10^4 epithelial cells per well of a

24-well plate, $\approx 2 \times 10^6$ midlogarithmic-phase bacteria ($OD_{600} = 0.4-0.6$) were added (i.e., ≈ 30 bacteria per epithelial cell), centrifuged onto the monolayer at $200 \times g$ for 5 min, and incubated for 2 hr at 37°C under 6% $\text{CO}_2/94\%$ air to allow invasion to occur. After this invasion period, the monolayer was washed twice with Earle's balanced salt solution (EBSS), and fresh prewarmed medium containing gentamicin at $100 \mu\text{g/ml}$ was added to kill extracellular bacteria. This treatment did not appear to affect the survival of intracellular bacteria. After another 2-hr incubation, the monolayer was washed twice with EBSS and lysed with 0.01% Triton X-100 in distilled water for 30 min (this treatment did not affect bacterial viability), and the released intracellular bacteria were enumerated by bacteriologic plate count. In control studies with equivalent bacterial numbers in the absence of epithelial cells, gentamicin at $100 \mu\text{g/ml}$ killed in 2 hr all bacterial strains used. Invasion ability was expressed as the percentage of the inoculum surviving the gentamicin treatment (i.e., recovery). Each assay was conducted in duplicate and was independently repeated at least three times. Results are expressed as an average of all replicate experiments.

Invasion Assays in the Presence of Biochemical Inhibitors. Requirements for invasion were studied by adding individual inhibitors to the monolayer either together with bacteria (i.e., chloramphenicol to inhibit bacterial protein synthesis) or 1 hr prior to the addition of bacteria (i.e., for inhibition of eukaryotic cell processes). Inhibitors were maintained throughout the 2-hr invasion period, and the assay was performed and the invasion ability was calculated as described above. The concentration of each inhibitor employed was chosen for maximal inhibitory effect without affecting epithelial cell viability over the assay period as measured by trypan blue (Hazleton Biologics, Lenexa, KS) staining. The bacteriostatic antibiotic chloramphenicol had no effect on monolayer or bacterial viability but did inhibit bacterial growth. The other inhibitors at the concentrations used were shown not to affect either bacterial viability or growth in cell culture media over the duration of the assay by comparing the number of bacteria after incubation with or without inhibitor.

RESULTS

Invasion Ability of Selected *Campylobacter* and *Citrobacter* Strains for Different Human Epithelial Cell Lines. Four *Campylobacter jejuni* strains and two *Citrobacter freundii* UTI isolates were compared in their ability to invade several different epithelial cell lines (Table 1). As previously reported (16), *S. typhi* Ty2 exhibited a 10% invasion frequency for INT407 cells, which was about 500-fold greater than the invasion ability of HB101 under these conditions. Of the four *Campylobacter jejuni* isolates examined, strains 81-176 (Table 1) and VC84 (1.5% invasion efficiency for INT407 cells; Table 2) were much more invasive than HB101 for all cell lines tested and showed a 70- to 50,000-fold higher recovery than reported earlier for other *Campylobacter jejuni* strains

Table 2. Effect of *de novo* protein synthesis inhibition on bacterial uptake into epithelial cells

Bacterial strains	% relative invasion of INT407 cells in presence and absence of inhibitor		
	Without inhibitor	With Cam at $100 \mu\text{g/ml}$	With Cyh at $20 \mu\text{g/ml}$
<i>Campylobacter jejuni</i> 81-176	100 (0.5)	2.5 ± 1.5	100 ± 25
VC84	100 (1.5)	1.1 ± 0.8	100 ± 23
<i>Citrobacter freundii</i> 3009	100 (0.7)	0.1 ± 0.08	100 ± 19
3056	100 (1.0)	0.6 ± 0.5	100 ± 15
<i>S. typhi</i> Ty2	100 (10.2)	0.4 ± 0.3	100 ± 22

Invasion assays conducted in the presence of chloramphenicol (Cam) or cycloheximide (Cyh) were compared to concurrent assays conducted without inhibitors. In the presence of Cyh at $20 \mu\text{g/ml}$, [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material of epithelial cells was only 10% of that observed with untreated cells (data not shown). Relative invasion in the presence of inhibitors is expressed as a percent of the invasion level achieved in the absence of inhibitors (i.e., 100%). Values shown are the average of three experiments, each conducted in duplicate \pm SEM. Numbers in parentheses, given only for assays conducted in the absence of inhibitors, are the actual percentage of input bacteria recovered intracellularly at the end of the assay period.

(10, 11), whereas strains A3249 and 3535 (data not shown) were 10- to 100-fold less invasive than HB101 (Table 1).

The two UTI *Citrobacter freundii* strains 3009 and 3056 invaded both bladder cell lines, T24 and 5637, at a 9- to 50-fold higher frequency than INT407 intestinal cells. To characterize the biochemical requirements for bacterial uptake into epithelial cells, invasion by *Campylobacter jejuni* was studied with INT407 cells, whereas *Citrobacter* invasion mechanisms were analyzed with both human intestinal (INT407) and bladder cell lines.

Inhibitor Analyses of Invasion Mechanisms. Effect of *de novo* protein synthesis inhibition. The inhibition of bacterial protein synthesis reduced by >97% the uptake into INT407 cells of both *Campylobacter* and *Citrobacter* as well as *S. typhi* Ty2 (Table 2). A comparable marked reduction of uptake of *Citrobacter freundii* strains into bladder cell lines was also observed (data not shown). In contrast, the inhibition of eukaryotic cell protein synthesis by cycloheximide (final concentration, $20 \mu\text{g/ml}$) had no effect on the uptake of these strains (Table 2).

Involvement of MFs. Cytochalasins, which have been shown to cause MF depolymerization (17), have been used to establish that *S. typhi* (and most other well-studied intracellular bacterial pathogens) use one or more MF-dependent pathways to enter epithelial cells (16, 18-21). Although uptake of *S. typhi* Ty2 in the presence of $2 \mu\text{M}$ cytochalasin D was inhibited >99% into INT407 cells and >94% into bladder cells, cytochalasin-mediated effects were both strain- and cell line-dependent for the *Campylobacter* and *Citrobacter* strains studied. Unexpectedly, the invasion ability of *Campylobacter jejuni* 81-176 for INT407 cells (Fig. 1) as well

Table 1. Invasion frequencies of selected bacterial strains for different human epithelial cell lines

Cell lines	% invasion*				
	<i>Campylobacter jejuni</i> 81-176	<i>Citrobacter freundii</i> 3009 3056		<i>S. typhi</i> Ty2	<i>E. coli</i> HB101
INT407	0.5 ± 0.2	0.7 ± 0.6	1.0 ± 0.9	10.2 ± 8.5	0.02 ± 0.01
T24	ND	12.8 ± 3.6	9.3 ± 6.7	6.3 ± 1.2	0.02 ± 0.01
5637	ND	35.0 ± 12.8	8.9 ± 3.8	4.0 ± 1.6	0.04 ± 0.01
HeLa	1.5 ± 0.2	ND	ND	6.3 ± 1.5	0.03 ± 0.02
HCT-8	0.2 ± 0.01	1.0 ± 0.5	8.4 ± 5.9	8.4 ± 1.8	0.07 ± 0.05
A-498	0.6 ± 0.1	0.4 ± 0.2	3.3 ± 2.1	7.9 ± 4.1	0.06 ± 0.02

*% invasion = percentage of input bacteria recovered after Triton lysis and represents the average of at least three separate experiments \pm SEM. ND, not determined.

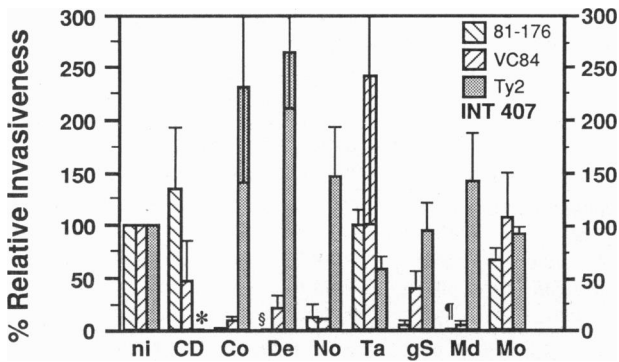


FIG. 1. Effects of various inhibitors on *Campylobacter* uptake into the INT407 cell line. One hour prior to the addition of bacteria to the monolayer, the epithelial cells were incubated either with no inhibitor (ni) or with 2 μ M cytochalasin D (CD), 10 μ M colchicine (Co), 1 μ M demecolcine (De), 20 μ M nocodazole (No), 50 μ M taxol (Ta), 250 μ M G-strophanthin (gS), 250 μ M monodansylcadaverine (Md), or 40 μ M monensin (Mo). Each inhibitor was maintained throughout the 2-hr invasion period. Relative percent invasiveness for each strain was determined as the recovery in the presence of inhibitors divided by the recovery (i.e., 100% relative invasiveness) observed in the absence of inhibitors. *S. typhi* Ty2 served as a consistent internal reference control. Results are presented as the mean (% relative invasiveness) of at least three different experiments \pm SEM, shown as bars above or below the means. Symbols *, \S , and \ddagger mark very low relative invasion values of $0.5 \pm 0.3\%$, $0.7 \pm 0.6\%$, and $0.3 \pm 0.2\%$, respectively.

as uptake of *Citrobacter freundii* into T24 cells (Fig. 2B) were not reduced by cytochalasin D (i.e., did not require MFs).

In contrast to *Campylobacter jejuni* 81-176, strain VC84 uptake into INT407 cells was reduced 50% by MF depolymerization (Fig. 1). Also, *Citrobacter*-mediated invasion of INT407 (Fig. 2A) or the 5637 bladder cell lines (Fig. 2C) was reduced >93% and >97%, respectively, by cytochalasin D. Thus, although *S. typhi* uptake was blocked by cytochalasin D in all cell lines, *Citrobacter* invasion was affected only in two of the three cell lines employed in this study (Fig. 2).

Involvement of MTs. To establish any involvement of MTs in the invasion mechanism(s), inhibitors that cause MT depolymerization or that stabilize existing MTs were used in invasion assays. The invasive ability of the *Campylobacter* strains was reduced >80% by MT depolymerization (Fig. 1). Also, the *Citrobacter* isolates invaded all three cell lines treated with MT-depolymerizing agents at a dramatically lowered frequency (Fig. 2). The MT-depolymerizing agents vincristine (50 μ M) and vinblastine (5 μ M) had similar but slightly less dramatic inhibitory effects (data not shown). In contrast, Ty2 invasion ability in general was not reduced by MT depolymerization (Figs. 1 and 2).

Stabilization of MTs in the presence of taxol had no inhibitory effect on INT407 uptake of *Campylobacter* (Fig. 1) or *Citrobacter* uptake into bladder cells (Fig. 2B and C). However, *Citrobacter* invasion of INT407 cells was reduced 48–72% by MT stabilization (Fig. 2A). *S. typhi* Ty2 invasion was reduced in half by taxol in all three cell lines (Fig. 2).

Coated-pit formation and bacterial endocytosis. The formation of coated-pits can be inhibited by g-strophanthin or monodansylcadaverine (29, 30). Preincubation of the epithelial cells with either compound at 250 μ M final concentration reduced the uptake of *Campylobacter* 81-176 to 4% and 0.3% respectively, and of VC84 to 40% and 4% respectively, of the no inhibitor control (Fig. 1). Similarly, all cell lines were reduced in their ability to engulf the *Citrobacter* isolates by these inhibitors of coated-pit formation (Fig. 2). In simultaneously run experiments, *S. typhi* Ty2 uptake was essentially unaffected by the presence of these inhibitors, with the exception of a 30% reduction in Ty2 relative invasiveness

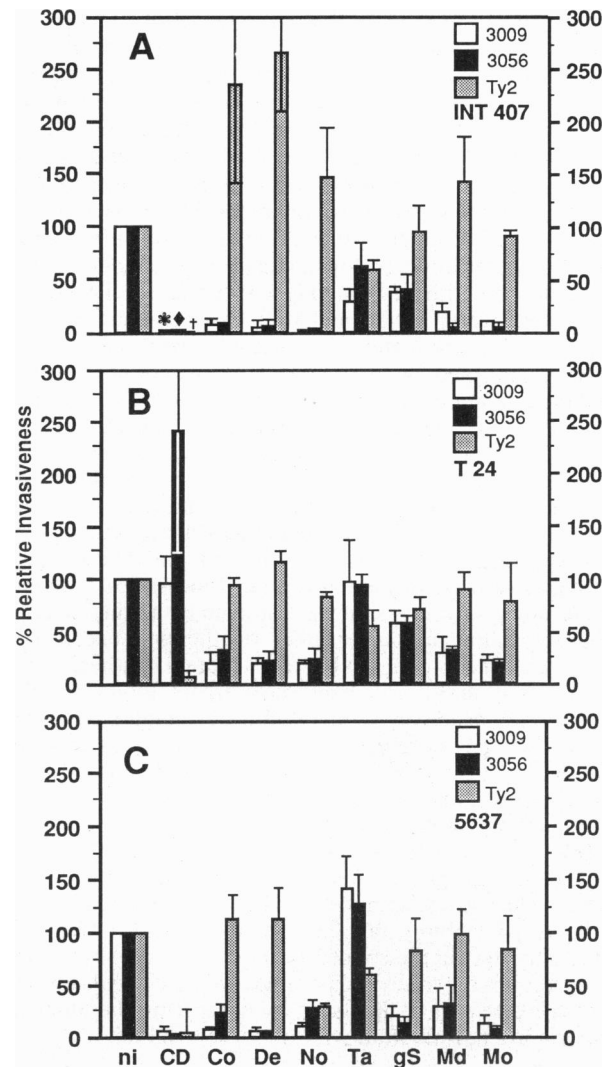


FIG. 2. Effects of various inhibitors on *Citrobacter* uptake into cells of intestinal (INT407) (A) or bladder (T24 and 5637) (B and C, respectively) origin. Invasion assays with or without inhibitor were performed as described in the legend to Fig. 1. *S. typhi* Ty2 served as a consistent internal reference control. Results are presented as the mean (% relative invasiveness) of at least three different experiments \pm SEM, shown as bars above or below the means. Symbols *, \diamond , and \ddagger denote low relative invasion values of $2 \pm 1.1\%$, $1.8 \pm 0.9\%$, and $0.5 \pm 0.3\%$, respectively.

into T24 cells in the presence of g-strophanthin (Fig. 2B). In control experiments, 250 μ M monodansylcadaverine blocked via coated pits the uptake of diphtheria toxin into INT407 cells and T24 cells as assessed by determining [35 S]methionine incorporation into protected cells (monodansylcadaverine added 1 hr prior to diphtheria toxin) and unprotected cells (no monodansylcadaverine added) in the presence of 10 nM diphtheria toxin as described by Moya et al. (22). The 35 S-incorporation rate was only 3% for unprotected cells versus 100% for protected cells in the presence of diphtheria toxin, compared with that seen in the absence of inhibitors (i.e., 100%).

Effect of endosome acidification on bacterial uptake/intracellular survival. Acidification of the endosome can affect eukaryotic receptor recycling to the cell surface (23, 24), potentially affecting bacterial entry, and can induce changes in engulfed bacteria that are necessary for intracellular survival (25). Because 20 mM NH_4Cl had only a slight effect and chloroquine at 40 μ g/ml reduced intracellular survival of all tested bacterial strains including Ty2, most

likely by accumulating intracellularly to bacteriocidal concentrations (21), these inhibitors were excluded from further studies. Therefore, to block endosome acidification, invasion assays were conducted solely in the presence of 40 μM monensin. *Campylobacter* uptake into INT407 cells was either not affected (i.e., strain VC84) or was reduced maximally $\approx 40\%$ (i.e., strain 81-176) under these conditions (Fig. 1). In contrast, *Citrobacter* invasion was reduced $\approx 80\text{--}90\%$ in all cell lines by blocking acidification with monensin, whereas Ty2 uptake appeared unaffected (Fig. 2). When *Citrobacter* invasion of T24 cells was carried out without inhibitor but monensin was added during the 2-hr gentamicin kill period, the number of intracellular bacteria was reduced only by 25% as compared with invasion ability in the absence of inhibitors. The decreased number of intracellular bacteria was apparently not due to reduced intracellular growth because *Citrobacter* was found not to replicate inside intestinal and bladder cells (data not shown). These results suggest that monensin may decrease intracellular *Citrobacter* survival to a small extent but has a large inhibitory effect on the initial entry event.

Electron Microscopic Analysis of *Campylobacter* and *Citrobacter* Endocytosis Events. Close apposition of *Campylobacter* to the surface of epithelial cells, and particularly their presence in the intercellular or junctional space (Fig. 3A),

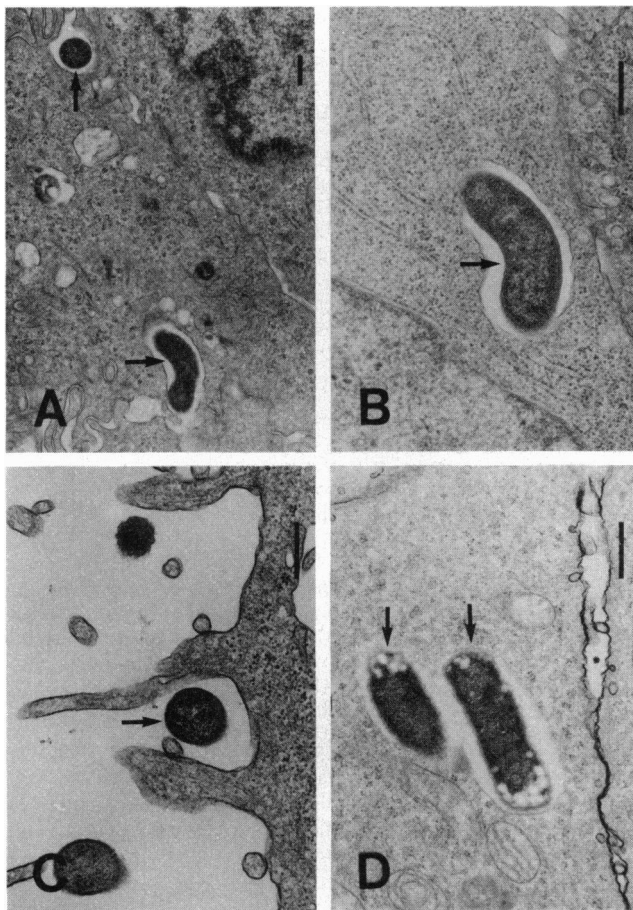


FIG. 3. Transmission electron micrographs of INT407 monolayers infected with *Campylobacter jejuni* 81-176 (A and B) and of T24 bladder cells infected with *Citrobacter freundii* 3009 (C and D). Bars in each micrograph represent 1 μm . Arrows point to bacteria interacting at the apical surface (C) or in intercellular spaces (A) or to bacteria that are contained within endosomal vacuoles (B and D). Micrographs A and C = 15-min time point, and micrographs B and D = 2-hr time point. Electron micrographic analyses for cells infected for various times were conducted essentially as described (16).

was noticed within 5 min of addition of bacteria to the monolayer. At later time points intracellular *Campylobacter* could be seen within endosomal vacuoles (Fig. 3B). *Citrobacter* or *S. typhi* Ty2 were never observed in the intercellular space with INT407 or T24 monolayers. Instead, *Citrobacter freundii* showed close interaction at the apical cell surface at early time points (Fig. 3C; note pseudopodia surrounding the bacterium), and after 30 min intracellular *Citrobacter* could be seen in endocytic vacuoles (Fig. 3D).

DISCUSSION

The epithelial cell endocytosis processes triggered by most invasive bacterial species that have been studied in detail are heavily MF-dependent, as defined by the pronounced inhibition of invasion caused by the only known MF depolymerizers, the cytochalasins. We describe here bacterial invasion mechanisms that are not inhibited by MF depolymerization. These distinctive mechanisms are most clearly illustrated by *Campylobacter jejuni* 81-176 uptake into INT407 cells and by *Citrobacter freundii* 3009 and 3056 entry into T24 bladder cells. Evidence for the strict MT dependence of these uptake processes is strengthened by the similar behavior of a series of inhibitors that act at different MT sites, plus the finding that concurrent Ty2 uptake was not reduced by MT depolymerization. Generally resulting in enhancement of Ty2 invasion ability into INT407 cells, MT depolymerization possibly relieves steric hindrance of the Ty2 MF-dependent uptake pathway. As one might expect, the stabilization of MTs with taxol allowed normal levels of invasion with these strict, MT-dependent pathways. These distinctive and analogous MT-dependent invasion processes, whose biochemical differences remain uncharacterized, exist in bacterial genera that are genetically quite distinct from one another.

Invasion ability was observed to be strongly dependent upon *de novo* bacterial protein synthesis for the *Campylobacter*, *Citrobacter*, and *S. typhi* Ty2 strains examined, as previously reported for *Salmonella choleraesuis* (26). In contrast, *de novo* protein synthesis is not required for *Yersinia enterocolitica* uptake (27). We propose that *Campylobacter*, *Citrobacter*, and *S. typhi* synthesize proteins of relatively short half-life (perhaps surface ligands for triggering uptake), which are essential for invasion.

Notwithstanding the MF-independent uptake described above, *Citrobacter freundii* invasion of INT407 or 5637 bladder cells was strongly MF-dependent and was also significantly reduced by MT depolymerization. Since these *Citrobacter* strains exhibit a strict MT-dependent uptake into T24 cells, it seems reasonable to hypothesize that these bacteria express two different invasion mechanisms [i.e., (i) MF-dependent and (ii) MT-dependent] in INT407 and 5637 cells. Expression of one or more bacterial-uptake pathways may be dependent upon the presence of different surface receptors in a particular epithelial cell line. Similarly, *Campylobacter jejuni* VC84 entry into INT407 cells was reduced about 2-fold by cytochalasin D and more markedly by MT depolymerization (Fig. 1), suggesting a lower efficiency MF-dependent mechanism for entry into INT407 cells in addition to a MT-dependent pathway. Alternatively, a more complex single uptake mechanism involving MFs and MTs could be involved.

Although nocodazole generally had no effect on Ty2 uptake into the different cell lines, this inhibitor, but not colchicine and demecolcine, consistently reduced Ty2 invasion of 5637 cells by 71%. This finding is difficult to reconcile mechanistically but might reflect (i) a colchicine/demecolcine-insensitive involvement of MTs in some step of the MF-dependent internalization pathway of Ty2 into 5637 cells or (ii) some uncharacterized action of nocodazole. In addition, taxol, which stabilizes MTs, consistently reduced Ty2

uptake by $\approx 40\%$ into all cell lines, suggesting (i) some involvement of MTs in the Ty2-uptake pathway or (ii) steric hindrance by polymerized MTs of the Ty2 MF-dependent uptake pathway. Likewise, we do not precisely understand the $\approx 50\%$ reduction of *Citrobacter freundii* uptake into INT407 cells caused by taxol. Speculatively, different mechanisms of MT-dependent uptake could be occurring in different cell lines, leading to this observation in INT407 cells. Despite these few problematic data interpretations, the consistent effects on bacterial uptake of many different chemical inhibitors of the same eukaryotic function plus the use of multiple cell lines have allowed us to reach decisive conclusions about the requirements for the *Campylobacter* and *Citrobacter* internalization pathways described here. Moreover, our findings demonstrate how the commonly employed method of using a single bacterial strain (often without positive or negative controls) to characterize bacterial uptake requirements can lead to an oversimplified or misleading view of bacterial internalization mechanisms.

The *Campylobacter jejuni* 81-176 MT-dependent uptake mechanism was inhibited $>98\%$ by two different inhibitors of coated-pit formation, indicating that clathrin-coated pits are somehow necessary for 81-176 endocytosis. The uptake of certain viruses or *Chlamydia* spp. by receptor-mediated endocytosis is thought to occur through receptors clustered in coated pits. Also, the uptake of strains VC84, 3009, and 3056 was reduced $>50\%$ by the two different inhibitors of coated-pit formation, suggesting possible involvement of coated pits in their invasion pathways. As stated earlier, inhibition of endosome acidification can potentially affect bacterial intracellular survival or uptake because of surface depletion of specific "invasion" receptors trapped in the unacidified endosome (reviewed in ref. 28). *Citrobacter freundii* uptake into all three cell lines was markedly impaired in the presence of monensin, suggesting a block in receptor recycling. There was no significant evidence that intracellular survival of *Campylobacter jejuni*, *Citrobacter freundii*, or *S. typhi* was dependent upon endosome acidification.

The above data suggest that for *Campylobacter* and *Citrobacter*, bacterial ligand-eukaryotic receptor interaction at the epithelial cell surface can trigger a MT-dependent membrane invagination event that leads to bacterial internalization. Subsequently, endosomes containing bacteria may be directed along MTs to specific intracellular sites. Further definition of the *Campylobacter* and *Citrobacter* genetic loci controlling uptake and additional electron and confocal microscopic examination of the involved epithelial cell events should clarify our understanding of these important processes.

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