

Penetration and Replication of *Edwardsiella* spp. in HEp-2 Cells

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The ability of 22 *Edwardsiella* strains to penetrate and replicate in cultured epithelial cells was initially evaluated by light microscopy methods and by the recovery of gentamicin-resistant (Gm^r) bacteria from the Triton X-100 cell lysates of HEp-2-infected monolayers. Giemsa-stained HEp-2 cells revealed the presence of numerous internalized bacteria 3 h postinfection, often appearing as parallel rows of replicated bacteria within the cytosol and sometimes obliterating the cytoplasm because of the large numbers of bacilli present. Invasive bacteria were also sometimes found within cytoplasmic vacuoles in infected cells; thin-section electron micrographs of HEp-2-infected cells supported these conclusions. Results of light microscopy studies and cell lysate assays indicated that most *Edwardsiella tarda* (92%) and some *Edwardsiella hoshinae* strains were invasion positive on one or more occasions, while *Edwardsiella ictaluri* isolates were uniformly negative. HEp-2 invasion by *E. tarda* was a microfilament-dependent (cytochalasin B- and D-sensitive) process, with maximum numbers of Gm^r CFU recorded between 3 and 6 h postinfection. The small percentage (0.01 to 1.0%) of the challenge inoculum recoverable as Gm^r progeny 3 to 6 h postinfection was attributed to a strong cell-associated (not filterable) hemolysin that was produced by a majority (85%) of the *E. tarda* strains but not by *E. ictaluri* and only minimally by *E. hoshinae*. This cytolysin/hemolysin was responsible for the toxic effects observed in HEp-2 cells during the infection-replication process of edwardsiellae and appears to play a role in the release of internalized and replicated bacteria from infected cells. The results suggest an invasion strategy with some similarities to and differences from those of other recognized enteroinvasive pathogens.

Edwardsiella tarda is the most commonly isolated member of the genus *Edwardsiella* and is the only species currently recognized to be pathogenic for humans (14). This species has a broad host range, having been isolated from fresh mussels, fish, reptiles, seals, swine, and other vertebrates (5, 11, 26, 31, 32). In humans, *E. tarda* has been most often associated with sporadic cases of diarrhea (1, 3, 19, 24), although a variety of extraintestinal diseases have been documented, including cellulitis, septicemia, meningitis, osteomyelitis, and hepatic abscesses (4, 15, 17, 20, 25, 27, 28, 34). Little information is available on what virulence factors may be operative in selected *E. tarda* infections or what elements regulate pathogenicity or host tropisms among the three currently recognized species comprising this genus.

Although still controversial, *E. tarda*-associated gastroenteritis can present in one of several clinical forms, the most common of which is an acute watery diarrhea resembling that produced by other toxigenic enteropathogens (3, 24). Less frequently, *E. tarda* has also been linked with dysenteric syndromes, sometimes resembling enterocolitis, in which macroscopic blood and fecal leukocytes are present in the feces of infected individuals (19). Two virulence factors compatible with these diarrheal syndromes have been reportedly produced by *E. tarda*: a heat-stable enterotoxin (2) and the ability to penetrate cultured epithelial cell monolayers (18). In the former instance, Bockemuhl et al. (2) in 1983 identified a heat-stable enterotoxin in the concentrated filtrate preparations of 3 of 25 *E. tarda* strains (12%) assayed either in the ligated rabbit ileal loop or in suckling mice; these studies have not been confirmed to date. In the case of invasion studies, results are also conflicting. Marques et al. (18) reported that all 12 clinical strains of *E. tarda* were invasive in HeLa cell assays, although only qualitative data

were presented. In contrast, Ullah and Arai (30) failed to detect any invasive characteristics in 19 *E. tarda* strains originating from eels and flounders in the same HeLa cell system. Furthermore, classic animal invasion models such as the keratoconjunctivitis (Sereny) assay performed in either rabbits or guinea pigs have been uniformly negative when *E. tarda* has been tested (2, 30). In this study, we report that most strains of *E. tarda* and some strains of *Edwardsiella hoshinae* are invasive in HEp-2 cells and that the former species produces a cell-associated cytolysin which is extremely active and makes performance and interpretation of invasion assays more difficult.

MATERIALS AND METHODS

Bacterial strains. Twenty-two *Edwardsiella* strains were studied: 13 isolates of *E. tarda*, 4 strains of *E. hoshinae*, and 5 isolates of *Edwardsiella ictaluri*. The sources and surface properties of these strains have been described previously (35). All *E. tarda* and *E. hoshinae* strains were grown at 35°C for invasion studies, while *E. ictaluri* isolates were cultured at 25°C. Stock cultures of each strain were maintained at ambient temperatures on extract agar slants during the course of this investigation.

HEp-2 invasion. (i) **Qualitative assays.** The invasive characteristics of all 22 *Edwardsiella* isolates were preliminarily screened in qualitative HEp-2 invasion assays. Each strain was grown overnight in brain heart infusion broth (BHIB); in the case of *E. ictaluri*, some strains required 48 h of incubation at 25°C to achieve suitable concentrations. After overnight growth, bacterial pellets were obtained by low-speed centrifugation of BHIB cultures; the supernatant was subsequently discarded, and the bacteria were then resuspended in phosphate-buffered saline (PBS; pH 7.2) and serially diluted to achieve a final inoculum of ca. 10⁶ CFU. HEp-2 cells were seeded into eight-well tissue culture cham-

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ber slides (Lab-Tek; Miles Laboratory, Naperville, Ill.) and grown in RPMI 1640 containing 10% fetal bovine serum with L-glutamine and antibiotics. Twenty-four hours prior to infection, the original growth medium was removed and replaced with RPMI 1640 lacking antibiotics. After removal of the antibiotic-free medium, each well of a slide chamber was inoculated with 200 μ l of RPMI 1640 (without antibiotics) containing 10^6 CFU of the challenge strain. The infection process was allowed to continue for 90 min at 35°C in a 5% CO₂ incubator, after which the inoculum was aspirated off and each well was washed 10 times in PBS. After the washing procedure, each well was overlaid with 300 μ l of RPMI 1640 containing fetal bovine serum, L-glutamine, gentamicin (50 μ g/ml), and lysozyme (300 μ g/ml) to kill any extracellular bacteria that had not penetrated HEp-2 cells and were not removed during the PBS washes (6, 7). Slide chambers containing this overlay solution were then reincubated in a CO₂ incubator for 3 h, after which the assay was terminated. The wells of each chamber were washed five times in PBS. One infected monolayer for each strain was then lysed in Triton X-100 (1%) for 5 min, after which aliquots of this suspension were plated onto heart infusion agar to determine the number of gentamicin-resistant (Gm^r) progeny (7). The other infected well was fixed in methanol and then stained with Giemsa stain and observed under light microscopy for evidence of HEp-2 invasion and replication of bacteria within the cytoplasm. *Escherichia coli* E20850/0 (O143:H⁻) and E20851/0 (O154:H⁻), both received courtesy of B. Rowe (London, England), and *Shigella dysenteriae* served as positive controls. Each strain was tested in a minimum of three independent experiments. Criteria used to assess invasive characteristics were as follows: light microscopy, Giemsa-stained HEp-2 cells displaying cytoplasm with five or more internalized bacteria in 1% of cells (16, 23); lysis assays, the recovery of more than 10^3 Gm^r CFU after Triton X-100 lysis. For each organism, a strain was considered positive in the respective assay (light microscopy or lysis) only if a minimum of two of three independent trials were positive.

(ii) **Quantitative assays.** Selected *Edwardsiella* strains were quantitatively evaluated for HEp-2 invasion as described above, with only slight modifications. Confluent monolayers of HEp-2 cells propagated in four-well tissue culture chamber slides were inoculated with 1×10^6 to 2×10^6 CFU of selected *Edwardsiella* strains grown overnight in BHIB. After the initial infection period, the inoculum was replaced by antibiotic-containing RPMI 1640. At various time intervals, this supernatant was removed from individual wells, and the remaining HEp-2 cells were lysed for 5 min in the presence of 1% Triton X-100 to release internalized (invaded) bacteria; the resulting Gm^r progeny were quantitated by duplicate plate counts of serial dilutions of each lysis supernatant.

Hemolytic activity. All 22 *Edwardsiella* strains were initially screened for hemolytic activity on heart infusion agar supplemented with 5% sheep erythrocytes; all plates were incubated for 72 h before a final reading was taken. For cell-free hemolytic activity, edwardsiellae were individually grown in BHIB overnight; the resulting cultures were then centrifuged at low speed to remove most of the intact cells and cellular debris before filtration of the supernatant fraction through 0.45- μ m-pore-size filters. These cell-free filtrates were then serially diluted in PBS and added to U-shaped microtiter plates, after which an equal volume (50 μ l) of a 1% (vol/vol) suspension of either sheep, rabbit, or guinea pig erythrocytes in PBS was mixed with each dilu-

TABLE 1. HEp-2 invasion by *Edwardsiella* species

Organism	No. tested	No. positive by ^a :		
		Light microscopy	Lysis assay	Both methods
<i>E. tarda</i>	13	3	0	5
<i>E. hoshinae</i>	4	0	0	1
<i>E. ictaluri</i>	5	0	0	0

^a Light microscopy, 5 or more bacteria per cell observed in >1% of HEp-2 cells; lysis, $\geq 10^3$ CFU of Gm^r bacteria recovered.

tion. Microtiter plates were then incubated for 1 h at 35°C before determination of the titer, which was the reciprocal of the highest dilution of cell-free filtrate that yielded 100% visual hemolysis. For cell-associated hemolytic activity, the bacterial pellets obtained from overnight BHIB cultures were resuspended in PBS and adjusted spectrophotometrically to an optical density at 610 nm of between 0.8 and 1.0. From this standardized suspension, twofold dilutions of bacteria in PBS were made; the assay was performed and hemolytic activity was determined as described for the cell-free system.

Inhibition of *E. tarda* HEp-2 invasion. To determine whether various inhibitors of receptor-mediated endocytosis, endosome acidification, or the eucaryotic cytoskeletal structure could prevent invasion by *E. tarda* ET-11 into HEp-2 cells, monolayers were preincubated for 45 min in the presence of various agents prior to infection as described by Finlay and Falkow (9, 10). After the infection process, the gentamicin-lysozyme-containing medium, which additionally contained the specific inhibitor, was added. After 3 h of incubation, the gentamicin-containing medium was removed, and HEp-2 cells were washed three times in PBS and then lysed with a 1% Triton X-100 solution. The effects of various inhibitors were then determined by measuring the ratio of Gm^r bacteria plus inhibitor to Gm^r bacteria without inhibitor; results were expressed as percentages.

RESULTS

Invasive characteristics. Preliminary results on the invasion of HEp-2 monolayers by *Edwardsiella* isolates indicated that infecting epithelial cells with initial inocula ranging between 4×10^5 and 4×10^6 CFU of each strain produced the best results for screening for invasive capabilities. Use of substantially higher concentrations of test organisms caused dramatic changes in HEp-2 cell morphology, ranging from cytotoxic reactions to total detachment of monolayers from slide culture chambers (see below).

The results of screening 22 *Edwardsiella* strains representing three distinct species for invasive capabilities in HEp-2 cells are shown in Table 1. Of the strains tested, nine (47%) were repeatedly positive in one or both assays by the criteria established. Of the nine positive strains, eight were identified as *E. tarda* (62%), while only one of four *E. hoshinae* (25%) and none of the five *E. ictaluri* gave similar responses. Of the 10 HEp-2-negative *Edwardsiella*, four strains (all *E. tarda*) were positive in one or both assays on at least one occasion, suggesting that these strains were invasive also; the only uniformly negative *E. tarda* strain studied was one serologically rough isolate, 3592-64.

On the basis of previous parameters established for other invasive organisms such as *E. coli* and *Shigella* spp. (6, 16, 23), we determined the number of Giemsa-stained HEp-2

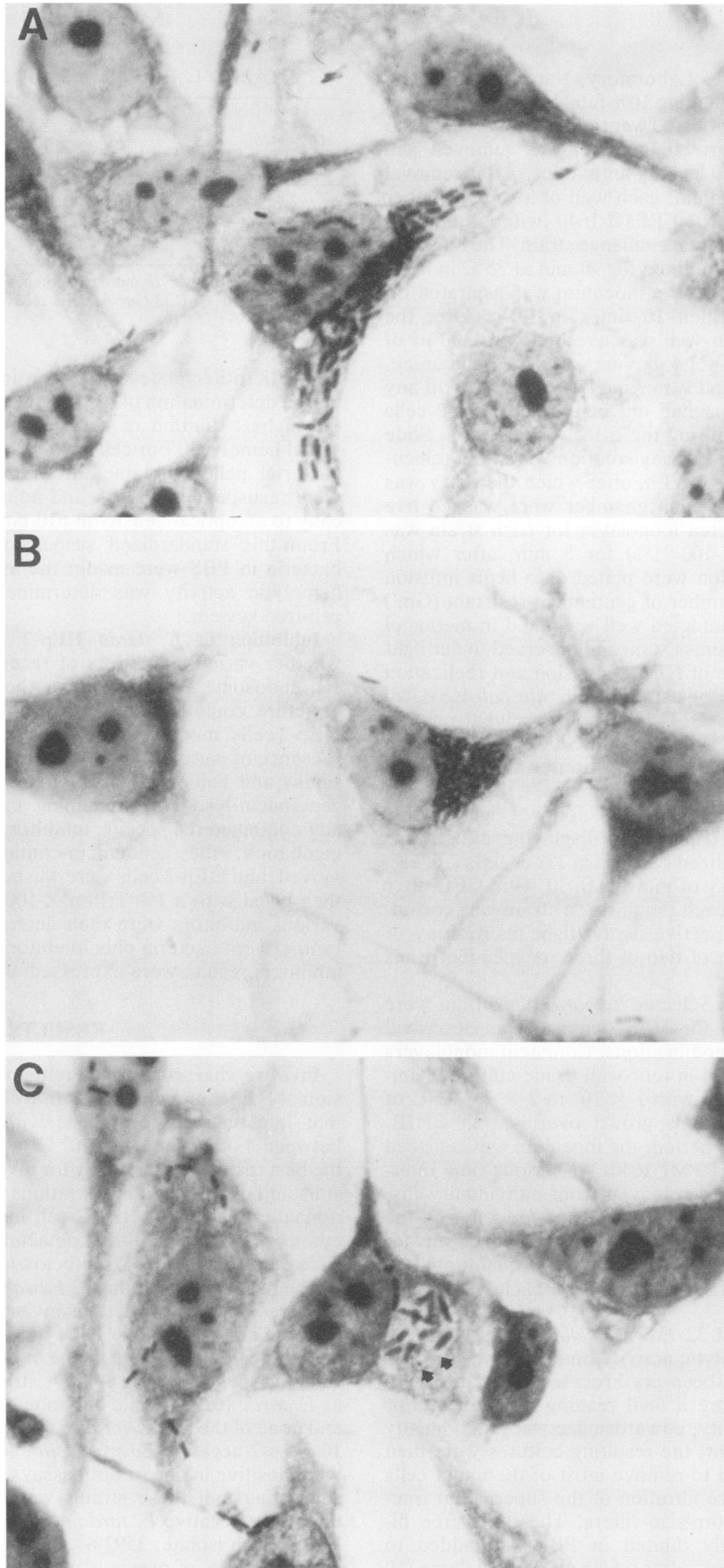


FIG. 1. Photomicrograph of the invasion and replication of *Edwardsiella* isolates in HEP-2 cells. Shown is invasion by *E. hoshinae* (A) and *E. tarda* (B); occasionally, bacilli enclosed in large vacuoles were observed (C).

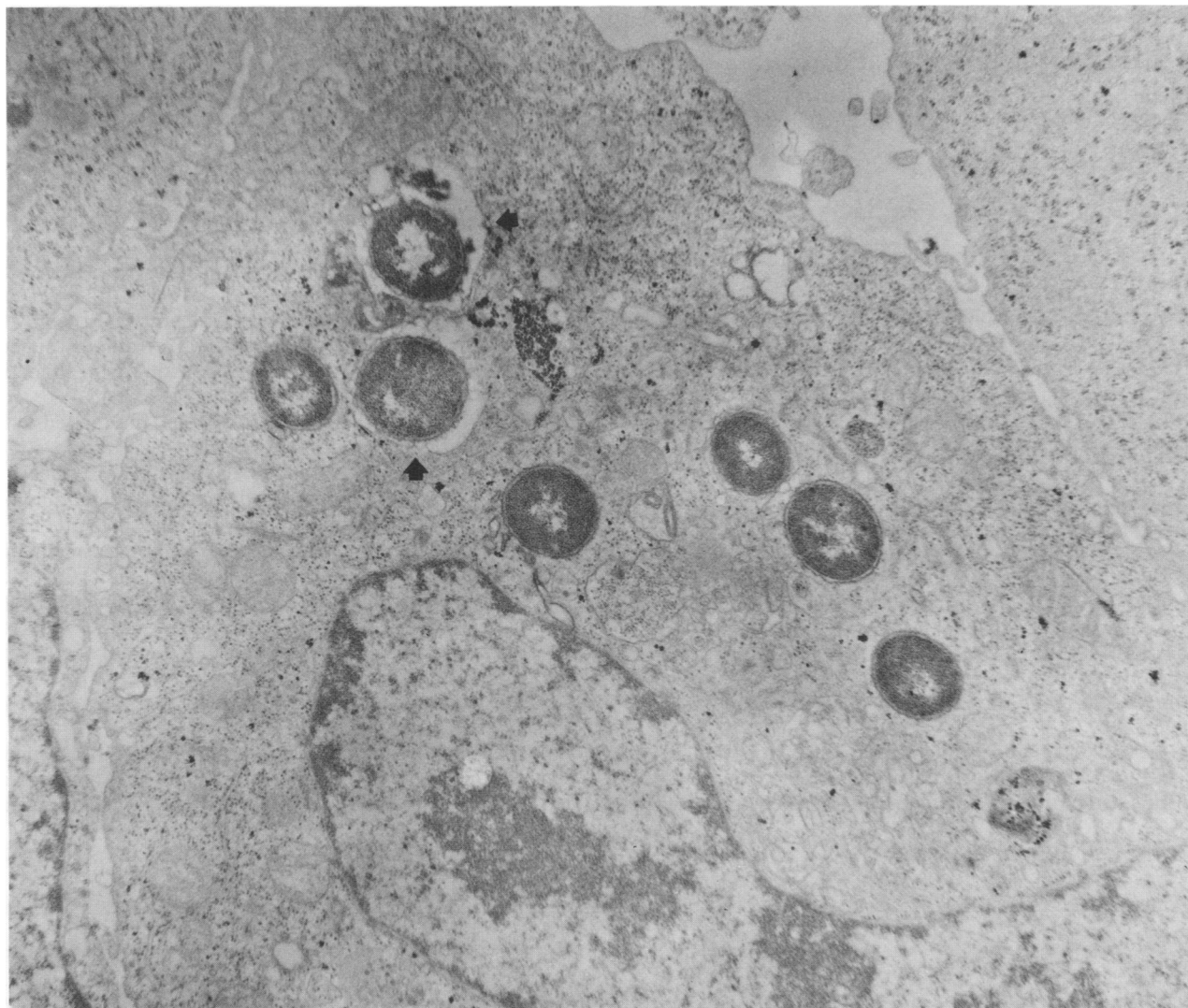


FIG. 2. Transmission electron micrograph of HEP-2 cells infected with *E. tarda* ET-11 3 h postinfection. Arrows indicate bacteria enclosed in vacuoles. Magnification, $\times 19,000$.

cells that contained five or more internalized bacteria per cell. Overall, between 1 and 8% of the HEP-2 cells from invasion-positive strains contained five or more bacteria per cell (mean for *E. tarda*, 0.4 bacteria per cell; minimum of 100 cells counted in 25 random fields). Invaded cells showed evidence of cellular replication of bacteria, as exemplified by the presence of multiple rows of parallel organisms within the HEP-2 cytoplasm as depicted for *E. hoshinae* 9-66 (Fig. 1A). In many instances, the number of intracellular bacteria was so large that individual bacterial morphology was almost completely obliterated by the massive quantities observed in the cytoplasm (Fig. 1B). Like some *E. coli* (23), occasional coccidial forms of *E. tarda* could apparently be seen in either the cytoplasm or nucleolus. Cells with large vacuoles containing many invasive bacteria were also noted (Fig. 1C). Thin sections of stained HEP-2 cells, when viewed by transmission electron microscopy, confirmed the presence of invaded edwardsiellae (Fig. 2). Some bacteria appeared to be enclosed within endocytic vacuoles, analogous to findings previously reported for *Salmonella* spp. and *Yersinia entero-*

colitica (10). Triton X-100 lysates of parallel-infected wells yielded between 10^2 and 10^4 CFU of Gm^r bacteria, which comprised between 0.01 and 1% of the initial inoculum; supernatants containing the gentamicin-lysozyme mixture assayed at various intervals during the replication phase were normally sterile or, on rare occasions, released one or two colonies of breakthrough growth. No *Edwardsiella* strain produced as high a percentage of infected HEP-2 cells or yielded as many Gm^r progeny as did any of the three control strains.

To more accurately quantitate the invasion process, three strains of *E. tarda* and two strains of *E. hoshinae* that had displayed various degrees of positivity in the previous assays (Table 1) were chosen for further study. After challenge with 10^6 CFU of individual strains, between 0.1 and 1% of the initial inoculum could be recovered in Triton X-100 cell lysates at 3 h postinfection in time course experiments; all strains except *E. tarda* SA8318 showed slight to moderate decreases in Gm^r progeny between 3 and 6 h postinfection, a 10-fold or greater decline in viable bacteria over the next

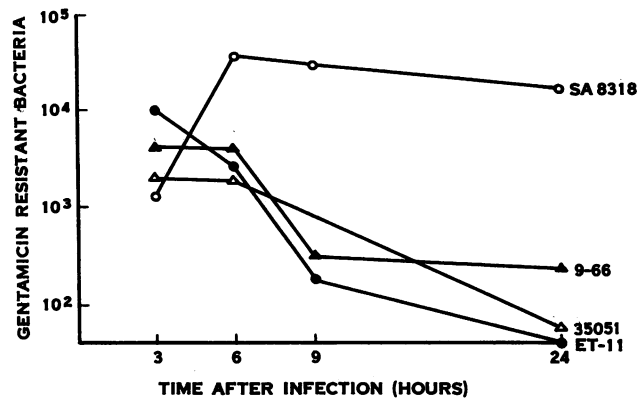


FIG. 3. Time course study of the number of recoverable Gm^F progeny from HEP-2 cells infected with either *E. tarda* ET-11 and SA8318 or *E. hoshinae* ATCC 35051 and 9-66.

3-h period dropping to less than 0.01% of the initial inoculum after 24 h. However, *E. hoshinae* ATCC 35051 and *E. tarda* ET-7 (data not shown), both negative in preliminary assays, were found to be invasive in time course experiments (Fig. 3). In no instance did invasive *Edwardsiella* strains show linear increases in recoverable Gm^F progeny from postinfection cell lysates similar to those reported for such organisms as *Salmonella choleraesuis*, *Y. enterocolitica*, and *Shigella flexneri* (10).

Cell-associated hemolysin. As previously mentioned, we noticed in the former assays a pronounced cytotoxic effect on HEP-2 cells during *Edwardsiella* invasion assays. This

toxic effect had been noted by previous investigators, who had postulated this effect to be due to a cytotoxin but could not demonstrate the identical effect by using cell-free culture filtrates (18). HEP-2 cells showing this toxic effect in our hands were often rounded with a darker nucleus and often exhibited extensive vacuolization in the cytoplasm. This effect could be observed as early as 30 min into the infection period. In some instances with high enough inocula, this toxic effect could cause almost complete detachment of confluent monolayers from tissue culture wells. The effect was associated with the bacteria themselves, since serial dilutions of challenge concentrations of four strains resulted in decreasing toxic effects to the monolayers, as noted by the residual number of Giemsa-stained cells remaining attached. Repeated washing of bacterial cells prior to the infection period did not alter this toxic effect.

To determine whether this apparent cell-associated cytolytic activity possessed hemolytic activity, all *Edwardsiella* strains were screened for lysis of sheep erythrocytes. Of the 13 *E. tarda* strains, 11 (85%) possessed a cell-associated hemolysin (≥ 4 hemolytic units) which was detectable neither in cell-free BHIB supernatants nor after prolonged incubation on sheep blood agar (Table 2). In only two minor instances (ET-8 and ET-11) could any unbound hemolysin be detected in cell-free supernatants and then only at the undiluted concentration. In contrast to *E. tarda*, hemolytic activity among *E. hoshinae* strains was extremely weak (mean, 65.1 versus 2.7), and we could not detect a similar enzymatic property in *E. ictaluri* isolates.

To determine whether the low number of Gm^F progeny detected in whole-cell lysates from previous experiments could be explained by the release of replicated bacteria from

TABLE 2. Hemolytic activities of *Edwardsiella* species

Organism	Strain	Source	Hemolytic activity ^a				
			Cell free ^b	Cell associated ^c			
				Rb	Shp	Gp	
<i>E. tarda</i>	15947	Feces	0	128	32	128	
	3592-64	Unknown	0	4	4	4	
	TK8403	Eel	0	0	0	0	
	SA8318	Flounder	0	2	0	2	
	F-1	Water	0	64	64	64	
	ET-2	Feces	0	128	64	128	
	ET-4	Feces	0	NT	128	NT	
	ET-6	Feces	0	NT	64	NT	
	ET-7	Feces	0	64	16	32	
	ET-8	Feces	2	NT	128	NT	
	ET-11	Feces	2	64	64	128	
	ET-12	Feces	0	64	128	64	
	ET-15	Blood	0	64	32	128	
	<i>E. hoshinae</i>	35051	Lizard	0	0	2	0
		1-78	Puffin	0	2	4	4
9-66		Lizard	0	4	2	4	
EH-1		Unknown	0	0	0	0	
<i>E. ictaluri</i>		33202	Catfish	0	0	0	0
	6006	Catfish	0	0	0	0	
	6012	Catfish	0	0	0	0	
	6013	Catfish	0	0	0	0	
	6017	Catfish	0	0	0	0	

^a No strain showed activity in the plate assay.

^b Maximum cell-free titer against rabbit, sheep, and guinea pig erythrocytes.

^c Against rabbit (Rb), sheep (Sp), and guinea pig (Gp) erythrocytes. NT, Not tested.

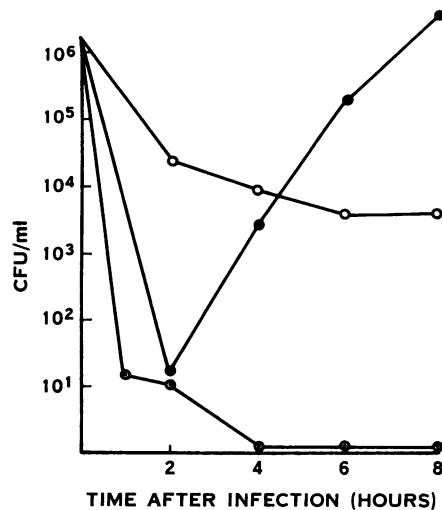


FIG. 4. Time course study of *E. tarda* ET-11 infection and release from HEp-2 cells; Symbols: ○, Triton X-100-released Gm⁺ bacteria; ○·, infectious progeny recovered from gentamicin-lysosome-containing supernatant; ●, infectious progeny recovered from HEp-2 supernatant from which all antibiotics were removed 1 h postinfection.

the cytoplasm of HEp-2 cells via the cell-associated hemolysin of *E. tarda*, we performed the following assay. Duplicate slide culture chambers containing confluent HEp-2 cell monolayers were simultaneously challenged with 10⁶ CFU of *E. tarda* ET-11 per well. At the end of the infection period, this medium was replaced by maintenance medium containing gentamicin and lysozyme. After a 1-h incubation, the supernatant from each well of one chamber slide was replaced by RPMI 1640 without antibiotics to allow for potential recovery of infectious progeny released from infected HEp-2 cells that would normally have been killed by exposure to the gentamicin solution. At 2 h postinfection, the only infectious bacteria recoverable were those obtained from lysis of HEp-2-infected monolayers (ca. 10⁴ CFU); both antibiotic-free and antibiotic containing media were essentially sterile (<10² CFU) (Fig. 4). At 4 h postinfection, large increases in CFU were detected in the antibiotic-free maintenance medium of HEp-2-infected monolayers. This increase continued over the remainder of the sampling period and reached 10⁶ CFU (initial inoculum) by 8 h postinfection.

Inhibitors of HEp-2 invasion. To determine the effects of various inhibitors on the invasion process of *E. tarda* ET-11 in HEp-2 cells, monolayers were preincubated with a variety of agents prior to challenge (Table 3). Inhibitors of receptor recycling (chloroquine) and of receptor-mediated endocytosis (dansylcadaverine) had no effect on HEp-2 invasion; neither did ammonium chloride, a lysosomotropic agent that inhibits endosome acidification, as does chloroquine. However, inhibitors of microfilaments (cytochalasins) and microtubules (colchicine) affected the invasion process. The reduction in HEp-2 invasion in the presence of colchicine increased with increasing concentrations of this agent.

DISCUSSION

One of the major virulence mechanisms by which gram-negative bacteria are thought to cause diarrheal disease is invasion of the gastrointestinal epithelium via specific chro-

TABLE 3. Effects of various inhibitors on *E. tarda* ET-11 HEp-2 invasion

Inhibitor	Concn	<i>E. tarda</i> invasion ^a
None		100
Chloroquine	200 µg/ml	98
Dansylcadaverine	500 µM	>100
Ammonium chloride	50 mM	>100
Cytochalasin B	5 µg/ml	41
Cytochalasin D	5 µg/ml	6
Colchicine	5 µg/ml	40

^a Gm⁺ bacteria plus inhibitor/Gm⁺ bacteria without inhibitor in HEp-2 cells, expressed as a percentage.

mosomal or plasmid-coded determinants (13). Major enteric pathogens shown to possess such mechanisms include most *Salmonella* species, *Shigella* spp., and some serogroups of *Y. enterocolitica* (7, 9, 10). This mechanism can be reproduced in vitro by demonstrating the ability of virulent strains to invade established mammalian cell lines such as HEp-2 or HeLa under defined conditions. Results of this investigation support the conclusions of Marques et al. (18) that most strains of *E. tarda* are indeed invasive in cultured monolayers; many strains of *E. hoshinae* appear to have similar invasive capabilities. In contrast, in the study of Ullah and Arai (30), invasive *E. tarda* were not detected by using similar techniques. While the reasons for this disagreement are unclear, several factors could explain these differences, such as the source of strains (environmental versus clinical) and the higher infecting inoculum (ca. 2 × 10⁷ in the latter study). Determination of whether the source and serotype of the isolate in question are linked to the invasion process will require further study.

This is, to our knowledge, the first report documenting *E. hoshinae*, as well as *E. tarda*, as an invasive species. At least two strains (ATCC 35051 and 9-66) possessed such capabilities, as detected in either the qualitative or quantitative assays. Very little is known about the pathogenicity of *E. hoshinae*, which is most commonly recovered from a number of different reptiles and other animals (12). However, it has been recovered from human feces on two occasions, and further studies should be conducted on this bacterium (8).

E. tarda invasion of epithelial cells appears to be a microfilament-dependent process, as indicated by the ability of both cytochalasins B and D to cause between a 60 and 95% reduction in the number of Gm⁺ progeny compared with controls; these values are similar to those reported for *Shigella*, *Salmonella*, and *Yersinia* invasion of MDCK cells by Finlay and Falkow (10). The potential effect of colchicine, a microtubule inhibitor, on *Edwardsiella* invasion requires further study. HEp-2 invasion was not inhibited by either chloroquine or dansylcadaverine, drugs known to inhibit receptor recycling and receptor-mediated endocytosis (including receptor-ligand clustering in coated pits) (10, 13). These findings parallel the results obtained with *Shigella flexneri* but contrast sharply with the invasion strategies employed by *Salmonella* and *Yersinia* spp., in which both agents markedly inhibit this process (10). This finding implies that both *Edwardsiella* and *Shigella* spp. penetrate and invade epithelial cells by a pathway with a group of receptors distinct from those utilized by either *Salmonella* or *Yersinia* spp. Finally, ammonium chloride, a lysosomotropic molecule that prevents endosome acidification (22), was ineffec-

tive in preventing *E. tarda* invasion, which agrees with results obtained for other gram-negative enteropathogens.

In addition, we have identified a cell-associated hemolysin similar to that produced by four animal strains of *E. tarda* as described by Watson and White (33) which appears responsible for the cytotoxiclike activity observed in eucaryotic cells used in invasion-attachment assays with this bacterium. This cytolysin/hemolysin was not detected in edwardsiellae incubated on agar plates containing sheep erythrocytes for prolonged periods of time; cell-free supernatants from broth-grown organisms contained less than 3% of the total hemolytic activity, indicating that it was apparently bound to the bacterium itself. This cell-associated hemolysin is phenotypically similar to the contact hemolytic activity of *Shigella* species which has been proposed to play a role in lysis of the phagocytic vacuole for release of virulent shigellae into the host cytoplasm, where replication occurs (29). One indirect line of evidence from this study suggests that the cell-associated hemolysin of *E. tarda* may play a similar role. *E. tarda* SA8318, originally recovered from a flounder, maintained stable levels of Gm^r progeny within infected cells from 6 to 24 h postinfection, unlike other strains tested (Fig. 3). This strain also failed to express any hemolytic activity against sheep erythrocytes in any of the three assays used, suggesting that failure to elaborate a cytolysin intracellularly prevented host cell lysis and release of infectious progeny into the gentamicin-containing supernatant. The results on detachment of HEp-2 cells from chamber slides and release of infectious progeny from ET-11-infected cells (Fig. 4) support this conclusion.

Of the major gram-negative enteric pathogens involved in invasive disease, the strategy utilized by *E. tarda* appears to most closely resemble that of *Shigella* spp., particularly in relationship to receptor-mediated endocytosis and cell-associated hemolytic activity (10, 13). However, there are several major differences between these genera. First, *E. tarda* is chemotactically motile, while *Shigella* spp. are not. Second, *Shigella* spp. produce a positive Sereny test, while *Edwardsiella* spp. lack this genetic locus (21). This locus, designated *kcpA*, appears to regulate spread of invasive bacteria from infected cells to adjacent cells. Finally, preliminary results do not suggest that large-molecular-weight plasmids are intimately associated with this invasion process (unpublished observations). Further studies defining specific similarities and differences in invasion strategies among enteric pathogens appear necessary in order to define molecular mechanisms and common virulence factors among these microbial agents.

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