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Expression of *Campylobacter jejuni* Invasiveness in Cell Cultures Coinfected with Other Bacteria

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Enteroinvasive *Salmonella*, *Shigella*, and *Escherichia coli* strains were found to exert an effect which rendered *Campylobacter jejuni* capable of intracellular localization in epithelial cells in vitro. When monolayers of HEp-2 or A-549 cells were challenged with pure cultures of *C. jejuni* or *Campylobacter coli*, none of the eight strains tested invaded the cells. In contrast, four of these strains were able to localize intracellularly when the cells were challenged with a mixture of campylobacters and enteroinvasive *Salmonella typhimurium*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, or *E. coli* strains. Invasiveness of campylobacters was also induced by one nonenteroinvasive strain of *E. coli* O124. Coinfection with other nonenteroinvasive *E. coli* strains did not induce invasiveness in *C. jejuni*. The degree of internalization induced by *S. typhimurium* was significantly higher than that induced by *Shigella* or *E. coli* strains. The invasive capacity of *C. jejuni* was found to differ considerably between strains. No evidence of an invasive potential was demonstrable for two *C. coli* strains or for two enterotoxigenic isolates of *C. jejuni* examined. *C. jejuni* was only able to localize intracellularly in cell cultures when the interaction occurred in a microaerobic atmosphere. None of the strains tested evoked keratoconjunctivitis in guinea pig eyes (Sereny test), regardless of the presence of coinfectants. The results indicate that a synergistic interaction that exists between *C. jejuni* and other enteropathogens facilitates invasion by *C. jejuni*.

The current interest in bacteria belonging to the genus *Campylobacter* started with the establishment of a causal relationship with acute diarrheal disease in humans (14, 15, 50). Two closely related species in this genus, *Campylobacter jejuni* and *Campylobacter coli*, are now recognized as important agents of diarrhea in both developed and developing countries (3, 15, 43, 44). However, the mechanisms by which these bacteria cause disease are incompletely understood. Invasiveness, enterotoxin production, and elaboration of cytotoxin have been suggested as potential pathogenicity factors (21, 25, 29, 30, 37, 40, 41, 43, 46). Evidence which indicates that a correlation exists between the pathogenic properties of specific isolates and the clinical status of the infected host has been presented (30). Accordingly, two distinct clinical presentations of *Campylobacter* enteritis have been distinguished: (i) an invasive-type bloody diarrhea and (ii) an enterotoxigenic-type watery diarrhea (30).

The invasive potential of *C. jejuni* has been established both in vitro and in vivo. Examination of biopsy specimens by histological, immunohistochemical, and electron microscopic methods has demonstrated that the pathogenesis of *Campylobacter* enteritis involves direct invasion of the intestinal mucosa (2, 16, 40, 51). Further evidence of an invasive capacity has been obtained from studies based on experimentally infected animals (40, 42, 45). Several research workers have examined the in vitro interaction between campylobacters and cell cultures. Although the ability of *C. jejuni* to localize intracellularly in cultured cells has been amply documented (15, 36, 40–43), the results have indicated substantial differences in invasive potential between strains (36, 40, 41). The development of quantitative assays of invasion is necessary to confirm these results (40). In the present study, we examined the ability of *C. jejuni* and *C. coli* to invade epithelial cells in vitro by using a combined light-optical method which enabled the quantitative assessment of intracellular bacteria (11). HEp-2 and HeLa cells have been successfully used to demonstrate a close correlation between the in vitro and in vivo invasiveness of *Salmonella* (12, 20, 26, 27), *Shigella* (12, 18, 19, 22, 23, 31, 38), and enteroinvasive *Escherichia coli* (EIEC) (17, 18) strains.

The mechanisms by which bacteria enter cells in vitro have been studied by several groups of investigators. Bukholm demonstrated that *Salmonella typhimurium* and *Shigella flexneri* were not able to enter cytochalasin B-treated HEp-2 cells (4), pointing to the active role of host cell microfilaments in this process. Coinfection of cell cultures with coxsackie virus B (8, 10) or measles virus (13) enhanced the invasiveness of *S. typhimurium* and *S. flexneri*. In HEp-2 and L-929 cells pretreated with interferons, the invasiveness of *S. typhimurium* was reduced (5, 7, 9). In a recent study, we showed that the probable mechanism for the interferon effect on the invasiveness of *S. typhimurium* is the inhibition of host cell endocytosis (6). All these data demonstrate that the invasiveness of salmonellae, shigellae, and EIEC is a process in which the host cell is the active agent: bacteria are endocytosed. Thus, in this context we use the words "invasive" and "invasiveness" merely as descriptive terms for the intracellular localization of bacteria in cell cultures. However, only bacteria carrying specific genetic information are endocytosed by epithelial cells (24, 26, 47, 48). In shigellae and EIEC this information is located in plasmids probably encoding polypeptides that induce receptor-mediated endocytosis (47). In salmonellae invasiveness genes are located in chromosomes.

It has been reported that *C. jejuni* is frequently encountered in mixed infections with other known bacterial enteropathogens (32, 39). This observation prompted us to study whether coinfection with other pathogens could influ-

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TABLE 1. *Campylobacter* strains studied

Species	Strain	Biotype[a]	Serotype	Clinical manifestation
C. jejuni	B/1	II	—[b]	Diarrhea
C. jejuni	27B-34	IV	PEN 27	Bloody diarrhea
C. jejuni	27B-22	IV	PEN 25	Bloody diarrhea
C. jejuni	27B-26	I	LAU 20	Diarrhea without gross blood
C. jejuni	CO11B[c]	I	—	Diarrhea
C. jejuni	INN 73-83[c]	I	—	Diarrhea
C. coli	28A-13	I	LAU 5,8	Diarrhea
C. coli	28B-3	II	LAU 13	Diarrhea

[a] According to Johnson and Lior (25).
[b] —, Unknown.
[c] Used as positive controls for enterotoxin production.

ence the interaction between *C. jejuni* and cultured cells. The results indicate that the ability of *C. jejuni* to localize intracellularly in epithelial cells is significantly enhanced by the presence of other enteropathogens as coinfectants.

MATERIALS AND METHODS

Bacterial strains. Six strains of *C. jejuni* and two strains of *C. coli* isolated from human clinical specimens were selected for study. Two of the *C. jejuni* strains examined were received from F. A. Klipstein (University of Rochester Medical Center, Rochester, N.Y.) as positive controls for enterotoxin production (INN 73-83 and CO11B). The enterotoxigenic potential of the remaining strains (our own isolates) was not known. All strains were biotyped by the methods and criteria of Lior (33), except that the DNA hydrolysis medium was changed to toluidine blue-DNA agar as recommended by Lior et al. (34). Serotyping was performed with heat-stable antigens by the scheme of Lauwers (28). The salient properties of the *Campylobacter* strains under study are presented in Table 1. Coinfection experiments were performed with the following 10 strains: *S. typhimurium* (SIFF S4575/81); *S. flexneri* serotype 4 (SIFF R662/81); *Shigella boydii* serotype 2 (SIFF S2783/81); *Shigella sonnei* (SIFF S3351/81); EIEC O124 (930-78); non-EIEC O124 (JH-13); and four nonenteropathogenic strains of *E. coli* (K-12 W1607, O142 C771, O147 G1253, and O150 1935). The two strains of *E. coli* O124 were kindly provided by K. Wachsmuth, Centers for Disease Control, Atlanta, Ga. All strains were maintained at −70°C. All the *Shigella* strains and the EIEC strain were invasive in HEp-2 cell monolayers and positive in the Sereny test. The *S. typhimurium* strain was invasive in HEp-2 cells but negative in the Sereny test. All the *Shigella*, *S. typhimurium*, and EIEC strains were originally isolated from patients with gastroenteritis.

Plasmid analysis. The *S. typhimurium*, *Shigella*, and *E. coli* strains were examined for the presence of virulence-associated plasmids by using a rapid small-scale modification of the alkaline lysis technique of Birnboim and Doly (1) as detailed by Maniatis et al. (35), followed by agarose gel (0.5%) electrophoresis of the resultant DNA preparations. Three strains of *E. coli* containing plasmids of known sizes (pDK9, Sa, and RP4) were routinely included as molecular weight standards on each gel.

Cultivation of cell monolayers. Monolayers of the human epithelial cell lines HEp-2 and A-549 were grown on glass cover slips (14 mm in diameter) in 24-well tissue culture plates (Linbro; Flow Laboratories, Inc., Inglewood, Calif.). Each well was supplied with 1 ml of cell suspension, approx. 10^5 cells, in Eagle basal medium (BME) containing bicarbonate, 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 μg/ml). The cells were incubated for 24 h at 37°C in a 5% CO_2 atmosphere with 100% relative humidity. The cell cultures were recognized as suitable for inoculation when they formed an almost continuous monolayer with intermediate blank areas (leopard spots). Prior to inoculation they were washed three times with 0.15 M phosphate-buffered saline (pH 7.4) (PBS) (37°C), incubated for 1 h in BME without antibiotics, and then washed three more times with PBS to minimize the concentrations of antibiotics. Finally, the cells were supplied with 1 ml of fresh BME containing 1% fetal bovine serum and no antibiotics.

Preparation of bacterial inocula. *Campylobacter* strains were prepared for testing by cultivation on chocolate agar at 42°C for 24 h under microaerobic conditions by using the GasPak system (code no. BR 38; BBL Microbiology Systems, Cockeysville, Md.) without a catalyzer. The bacteria were harvested in PBS, and the concentration was adjusted to an optical density of 1.0 at 520 nm in a Hitachi spectrophotometer (model 101). *S. typhimurium*, *Shigella*, and *E. coli* strains were cultivated on chocolate agar for 24 h at 37°C under aerobic conditions. The bacteria were suspended in PBS to an optical density of 0.5 at 520 nm, corresponding to approximately 3.5×10^7 bacteria per ml.

Preparation of filtrates. Three types of filtrates were prepared. (i) Overnight growth of *S. typhimurium* (SIFF S4575/81) on chocolate agar was harvested in PBS to an optical density of 0.75 at 520 nm, and the suspension was passed through a sterile membrane filter with a pore size of 0.32 μm (Millipore Corp., Bedford, Mass.). (ii) Monolayers of HEp-2 and A-549 cells were inoculated with 100 μl of *S. typhimurium* suspension and incubated for 12 h at 37°C in a microaerobic atmosphere. The cells containing bacteria were scraped off the cover slip, and the resultant suspensions were centrifuged at 3,000 × *g* for 10 min. In parallel set-ups the suspensions were sonicated by two 10-s bursts (20 kHz, 50 W) from a model B12 ultrasonic disintegrator (Branson Sonic Power Co., Danbury, Conn.) before centrifugation. The supernatants were filtered through sterile 0.32-μm-pore-size membrane filters. The filtrates were either dialyzed against fresh BME with 1% fetal bovine serum for 24 h to restore pH and salts or used without dialysis. (iii) Monolayers of HEp-2 and A-549 cells were inoculated with 50 μl of *S. typhimurium* and 100 μl of *C. jejuni* (B/1) suspensions and incubated for 12 h in a microaerophilic atmosphere. Sterile filtrates were prepared with or without sonication and dialysis as described above.

Challenge of cell monolayers. The cell monolayers were challenged with five different types of inocula: (i) 100 μl of *Campylobacter* suspension: (ii) 100 μl of *Campylobacter* suspension plus 50 μl of *S. typhimurium*, shigellae, or *E. coli*; (iii) 100 μl of *Campylobacter* suspension plus 200 μl of sterile *S. typhimurium* filtrate; (iv) 100 μl of *Campylobacter* suspension plus 200 μl of nondialyzed filtrates from cell cultures infected with *S. typhimurium* or *S. typhimurium* plus campylobacters; and (v) 100 μl of *Campylobacter* suspension plus 1 ml of freshly dialyzed filtrates from cell cultures infected with *S. typhimurium* or campylobacters plus *S. typhimurium*. In the above-mentioned experiments, the cell culture medium (BME) was removed from the cell monolayers and replaced with the dialysates before inoculation with campylobacters. Controls were provided by inoculating the cells with pure cultures of *S. typhimurium*, shigellae, or *E. coli*. After inoculation, the cells were incu-

bated under three different atmospheric conditions: (i) 5% CO_2, (ii) microaerobic atmosphere, or (iii) aerobic atmosphere. All incubations were performed at 37°C in saturated humidity for 5, 8, or 12 h. Experiments involving inocula (i) and (ii) were repeated five times on separate days.

Fixation and staining. Before fixation, the cells were washed three times with PBS to remove bacteria not associated with the cells. The cells were then fixed overnight with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After fixation, the cells were washed three times in the same buffer to remove glutaraldehyde. The specimens were stained for 4 min with acridine orange (50 mg/liter) (12) in cacodylate buffer, washed three times in the buffer, and mounted on glass slides with a drop of immersion oil for microscopy.

Microscopy. The preparations were examined at a final magnification of ×1,250 by using a combination of two optical systems applied on the same microscope (Nikon): (i) Nomarski differential interference contrast microscopy and (ii) UV incident-light microscopy. A detailed description of the methods used has been done by Bukholm et al. (11). This approach enabled effective discrimination between extracellular (adhesive) and intracellular (invasive) bacteria. The accuracy and sensitivity of this approach have been established by comparison with scanning electron microscopy (11). Bacteria that were intracellularly located by this procedure were described as invasive bacteria in this study. The term invasive is thus only used as a descriptive term and does not refer to any speculations about the mechanisms by which the bacteria enter the cells. About 200 cells in each of three parallel preparations were examined for intracellular campylobacters. The campylobacters were differentiated from the other gram-negative rods by their special morphology. From these data the following parameters were calculated: (i) the percentage of cells containing intracellular bacteria and (ii) the mean number of intracellular bacteria per infected cell. Statistical analyses were based on the Pearson chi-square test applied to the different frequency distributions of cells with intracellular bacteria. P values below 0.05 were regarded as significant.

Sereny test. Bacterial suspensions were prepared in exactly the same way as the bacteria used to inoculate the cell cultures. Equal volumes of *Campylobacter* and *S. typhimurium* suspensions were mixed together, and 0.1-ml aliquots were deposited into the conjunctival sac of one guinea pig eye. Controls included animals challenged with pure cultures of campylobacters or *S. typhimurium*. The guinea pigs were observed daily for 7 days for the development of keratoconjunctivitis (49).

RESULTS

Inoculation with *Campylobacter* pure cultures. The eight *Campylobacter* clinical isolates were initially tested for their ability to invade HEp-2 or A-549 cells in the absence of coinfectants. When the cell monolayers were challenged with *Campylobacter* pure cultures, intracellular bacteria were never observed for incubations up to 12 h, although extracellular bacteria were occasionally detected. The results obtained after incubation of the cells under three different atmospheric conditions (i.e., aerobic, 5% CO_2, or microaerobic) were identical, regardless of the cell line used.

Coinfection with *S. typhimurium*. We next examined whether the nature of the interaction between *C. jejuni* or *C. coli* and the cell cultures was affected by the presence of *S. typhimurium* as a coinfectant. This was, indeed, found to be the case. When cells were challenged with a mixture of *C. jejuni* and *S. typhimurium* SIFF S4575/81, four of the eight *Campylobacter* strains tested proved to be capable of intracellular localization in both HEp-2 and A-549 cells. All four intracellular strains were *C. jejuni*, whereas the two strains of *C. coli* included in this study remained extracellular for incubation times up to 12 h. Likewise, two *C. jejuni* strains originally received as positive controls for enterotoxin production (INN 73-83 and C011B) were not localized intracellularly. The number of intracellular bacteria and infected cells varied significantly between the strains (Table 2). To facilitate comparison, we examined all *Campylobacter* strains on the same day and used the same *S. typhimurium* inoculum. After 8 h of incubation, the percentage of cells with intracellular bacteria ranged from 2.3 to 61.3%, and the mean number of intracellular bacteria per infected cell varied from 2 to 20. The highest degree of internalization was exhibited by strain B/1, which attacked 100% of the cells with more than 20 bacteria per cell after 12 h of incubation. It is notable that this strain had been subcultured several times in the laboratory, whereas the other strains tested had been subjected to relatively few passages on artificial media, indicating that the invasive potential was not lost after subcultivation.

TABLE 2. Invasiveness of *Campylobacter* strains cocultured with *S. typhimurium* in A-549 cells[a]

Strains	% (Range) of cells with intracellar campylobacters	Mean no. (range) of intracellular campylobacters per infected cell
C. jejuni		
B/1	49.7 (43.5–61.3)	17.0 (15.2–22.3)
27B-34	15.0 (10.3–17.4)	4.9 (3.5–5.7)
27B-22	5.7 (3.7–9.5)	3.4 (2.3–4.6)
27B-26	3.5 (2.3–5.8)	3.2 (2.1–4.8)
CO11B	0	0
INN 73-83	0	0
C. coli		
28A-13	0	0
28B-3	0	0

[a] Data represent the mean values of 200 counted cells in each of three parallel experiments after incubation for 8 h. Data in parentheses represent the ranges obtained after five different experiments on five different days. Cells inoculated with *Campylobacter* pure cultures contained no intracellular campylobacters.

To examine whether the ability of *C. jejuni* to localize intracellularly was dependent upon the concomitant internalization of *S. typhimurium*, we recorded the frequency with which *S. typhimurium* and *C. jejuni* B/1 were encountered together in the same cell. Although 30% of the cells were invaded by both bacteria after 8 h of interaction, 14% harbored intracellular campylobacters only, and 2% contained only *S. typhimurium*. Moreover, the mean number of campylobacters per cell did not differ significantly between cells harboring only campylobacters (8.4) and cells containing both bacterial species (7.0) (Table 3).

Atmospheric conditions were found to be critical for the expression of invasiveness. Intracellular campylobacters were only observed after incubation in a microaerobic atmosphere. The ability to localize intracellularly was totally abolished when the cells were incubated in 5% CO_2 or air, even after 12 h of incubation. However, the invasiveness of *S. typhimurium*, shigellae, and *E. coli* was also expressed in aerobic and 5% CO_2 atmospheres.

TABLE 3. Relative distribution of intracellular *C. jejuni* and *S. typhimurium* in A-549 cell cultures[a]

Infectant		% of cells with intracellular bacteria		Mean no. of bacteria per infected cell	
S. typhimurium	*C. jejuni*	*S. typhimurium*	*C. jejuni*	*S. typhimurium*	*C. jejuni*
+	−	2	0	4	0
+	+	30	30	3.8	7.0
−	+	0	14	0	8.4

[a] Bacteria and cells were allowed to interact for 8 h.

Although there was no significant difference between HEp-2 and A-549 cells with regard to their susceptibility to invasion, it proved easier to perform the assay with A-549 cells than with HEp-2 cells because acridine orange staining enabled more effective discrimination between bacterial and cellular cytoplasmas when A-549 cells were used.

Coinfection with shigellae and *E. coli*. The observation that the ability of *C. jejuni* to invade cell cultures was drastically altered by the presence of *S. typhimurium* prompted us to study whether coinfection with other bacterial enteropathogens could induce an analogous effect. All studies were performed with *C. jejuni* B/1, and the cells (A-549) were incubated with bacteria under microaerobic conditions for 8 h. All coinfectants were examined on the same day with the same *C. jejuni* inoculum. Like *S. typhimurium*, the *S. flexneri*, *S. boydii*, and *S. sonnei* strains and the EIEC O124 strain were all found to render *C. jejuni* capable of invading the cells. However, the degree of internalization induced by the shigellae and *E. coli* was significantly lower than that induced by *S. typhimurium* (Table 4). All of these strains harbored high-molecular-weight plasmids which have been associated with the enteroinvasive potential of the bacterial species concerned (Table 4). On the other hand, one non-EIEC O124 strain, which lacked plasmids, was also found to induce the invasiveness of *C. jejuni*, indicating that this ability was not dependent on the presence of virulence-associated plasmids within the *Shigella-E. coli* group. Intracellular *C. jejuni* was not detected when the four nonenteropathogenic strains of *E. coli* were used as coinfectants, although one of the strains contained a 140-megadalton plasmid.

Effect of bacterial filtrates. To test the possibility that an extracellular substance was responsible for the observed activation of *C. jejuni* invasiveness, we inoculated HEp-2 and A-549 cells with a mixture of *C. jejuni* B/1 and a sterile filtrate of *S. typhimurium* SIFF S4575/81 suspended in PBS. The experiment was repeated with sterile filtrates from HEp-2 and A-549 cell cultures infected with *S. typhimurium* or *S. typhimurium* plus campylobacters. The cells were inoculated with campylobacters in the presence of freshly dialyzed filtrates. No intracellular campylobacters were detected in any of these experiments, regardless of the cell line used.

Test for in vivo invasiveness. Neither *C. jejuni* (28, 32) nor *S. typhimurium* (8) causes keratoconjunctivitis in guinea pig eyes (Sereny test). The data from the experiments described above encouraged speculation as to whether coinfection with *S. typhimurium* would enable *C. jejuni* to evoke a positive Sereny reaction. To test this hypothesis, we deposited all eight *Campylobacter* strains included in this study, either alone or with *S. typhimurium* SIFF S4575/81, onto guinea pig eyes. No significant reaction was demonstrable for any of the strains, regardless of the presence of *S. typhimurium*. Thus, we failed to establish a correlation between the ability of *C. jejuni* to invade epithelial cells in vitro and to evoke guinea pig keratoconjunctivitis.

DISCUSSION

The invasive potential of enteropathogenic bacteria is becoming increasingly recognized as an important determinant of virulence. In addition to animal models, in vitro tissue culture assays have been used to study the invasive capacity of enteric pathogens. However, efforts to obtain conclusive experimental evidence have been hampered by difficulties in differentiating between extracellular and intracellular bacteria. The results obtained from studies assessing the interaction between *C. jejuni* and cell cultures are not uniform (36, 40, 42). The use of different techniques and criteria for invasiveness may, at least partly, explain the observed variability. In the present study we used a quantitative light-optical method which enabled effective discrimination between extracellular and intracellular localizations of bacteria (11). Some of the strains examined were isolated from patients with fresh blood in their stools (Table 1), indicating that invasion was the most likely pathogenic mechanism. Despite this, none of the strains tested were able to localize intracellularly in HEp-2 or A-549 cells when the cells were challenged with pure cultures of campylobacters. In contrast, when the campylobacters were allowed to interact with cells in the presence of other enteropathogenic bacteria, the invasive potential was expressed. Certain *S. typhimurium*, *Shigella*, and *E. coli* strains seemed to exert an effect which rendered *C. jejuni* capable of intracellular localization. This in vitro observation parallels the general observation that *C. jejuni* is frequently encountered in mixed infections with other enteropathogenic bacteria. Indeed, some authors have reported a surprisingly high incidence of mixed infections with *C. jejuni* as compared with those involving other enteropathogenic bacteria (32, 39). These findings encourage speculation as to whether a synergistic interaction exists between *C. jejuni* and other pathogens and facilitates the invasion of *C. jejuni* in vivo. However, despite

TABLE 4. Invasiveness of *C. jejuni* B/1 in A-549 cells coinfected with *S. typhimurium*, shigellae, or *E. coli*[a]

Coinfectant	Plasmid (megadaltons)	Sereny test result	Invasiveness[b]	% of infected cells	Mean no. of campylobacters per infected cell
S. typhimurium SIFF S4575/81	62	−	245	49.7	17.0
S. flexneri SIFF S662/81	140	+	270	10.2	4.1
S. boydii SIFF S2783/81	140	+	33	8.8	3.5
S. sonnei SIFF S3351/81	120	+	20	8.1	3.9
E. coli O124 930-78	140	+	250	11.3	3.5
E. coli O124 JH-13	None	−	0	9.2	3.7
E. coli K-12 W1607	None	−	0	0	0
E. coli O142 C771	100	−	0	0	0
E. coli O147 G1253	120	−	0	0	0
E. coli O150 1935	140	−	120	0	0

[a] Bacteria and cells were allowed to interact for 8 h.
[b] Mean number of bacteria per 100 A-549 cells.

the in vitro evidence, we failed to demonstrate the internalization of *C. jejuni* in guinea pig corneal cells (Sereny test), regardless of the presence of coinfectants. It is notable, though, that *C. jejuni* was able to invade cell cultures only when the interaction occurred in a microaerobic atmosphere. Exposure to aerobic conditions, like those experienced on the surface of guinea pig eyes, resulted in suppression of the invasive potential. Thus, the possibility remains that coinfection with other pathogens increases the ability of *C. jejuni* to invade epithelial cells in the human intestinal tract, where the bacteria are likely to encounter reduced oxygen tension.

The present study supports previously published data indicating that the invasive capacity of *C. jejuni* differs significantly between strains (36, 40, 41). Of the eight *Campylobacter* strains tested, only four were able to invade epithelial cells in our in vitro model. No evidence of invasive potential was detectable for either of the two *C. coli* strains under study. Examination of a more comprehensive strain collection will be needed to determine whether *C. jejuni* and *C. coli* differ in their ability to invade epithelial cells and to ascertain whether any correlation exists between invasiveness and the biotype, serotype, or ecological origin of the strains. The results presented by Klipstein et al. (30) suggest that invasiveness and enterotoxin production reflect two pathogenic strategies of *C. jejuni* which are responsible for distinct clinical forms of *Campylobacter* enteritis. Although two enterotoxigenic strains were noninvasive in our in vitro assay, the present study is too limited to warrant conclusions as to a potential association among invasiveness, enterotoxin production, and the clinical status of the infected host.

The observation that sterile filtrates from cell cultures inoculated with *S. typhimurium* or *S. typhimurium* plus campylobacters were not capable of inducing *C. jejuni* internalization strongly suggests that the invasion-facilitating effect was not caused by an extracellular filterable substance. Direct contact between the coinfectants and the cells would be another explanation. However, internalization of the coinfectants was not required for the uptake of campylobacters, since many cells were found to contain campylobacters only. Furthermore, *E. coli* JH-13 was capable of inducing *C. jejuni* invasion even though it was completely noninvasive itself (Table 4). We could not demonstrate any correlation between the number of intracellular coinfectants and the number of intracellular campylobacters. The mean number of campylobacters per cell did not differ significantly between cells containing both campylobacters and coinfectants and cells harboring campylobacters only. Although the coinfectant that most readily facilitated the entrance of campylobacters was highly invasive itself, a similar correlation was not demonstrated for the other coinfectants under study (Table 4). Indeed, no intracellular campylobacters could be detected when *E. coli* 1935, which was highly invasive, was used as the coinfectant.

However, the mechanisms for the interaction are still obscure. We do not know whether the effect exerted by the coinfectants is directed at the host cell or the campylobacters. Bacterial invasiveness is usually a host cell-dependent process. However, the fact that invasiveness was only expressed under atmospheric conditions that rendered campylobacters capable of growth points to the necessity of an active campylobacter metabolism. In fact, the invasiveness of campylobacters could be selectively blocked by increasing the O_2 tension; the coinfecting salmonellae or shigellae were still invasive. These results point to a mechanism of invasiveness different from that of *S. typhimurium*, which is able to localize intracellularly even when the bacteria are killed by gentamicin treatment (50 μg/ml; data not shown). However, the mechanisms for campylobacter entry into the cells have to be elucidated by further studies.

The other question is why only certain coinfectants are able to induce this internalization process. Enteroinvasive *Shigella* strains and EIEC harbor virulence-associated plasmids of 120 to 140 megadaltons (24, 47, 48). The presence of these plasmids is a prerequisite for the expression of invasiveness in tissue cultures in vitro (47). Besides plasmid-controlled functions, chromosomal determinants are also required for in vivo virulence and for positive Sereny test (47). The effect observed in the present study seemed to reflect a synergistic interaction between *S. typhimurium*, shigellae, or EIEC on the one hand and *C. jejuni* on the other. All these strains were capable of invading HEp-2 cells by themselves; the *Shigella* and EIEC O124 strains harbored 120- to 140-megadalton virulence-associated plasmids. However, two strains were exceptions: (i) one *E. coli* O124 strain lacked the plasmid, was noninvasive, but was still able to induce the internalization of *C. jejuni*, and (ii) one nonenteropathogenic *E. coli* strain harbored a 140-megadalton plasmid, was invasive, but was unable to induce the internalization of *C. jejuni*. The latter strain was also negative in the Sereny test (Table 4), indicating that the chromosomal genes necessary for in vivo virulence were lacking.

In conclusion, enteroinvasive bacteria exerted an effect in cell cultures that made some strains of *C. jejuni* invasive. The same effect was achieved by cocultivation with an *E. coli* O124 strain that was noninvasive and lacked the virulence-associated plasmid. The invasiveness of *C. jejuni* could be separately blocked by increasing the O_2 tension in the media, indicating that *C. jejuni* metabolism was essential for invasiveness. However, the mechanisms remain unclear. Research aimed at elucidating these questions may increase our understanding of the little-known determinants of bacterial synergism.

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