INFECTION AND IMMUNITY, July 1987, p. 1674–1679
0019-9567/87/071674-06$02.00/0

Vol. 55, No. 7

Comparison of the Ability of Enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to Enter and Replicate within HEp-2 Cells

PAMELA L. C. SMALL,[1]†* RALPH R. ISBERG,[2] AND STANLEY FALKOW[1]

Department of Medical Microbiology, Stanford Medical School, Stanford, California 94305,[1] and Department of Molecular Biology and Microbiology, Tufts School of Medicine, Boston, Massachusetts 02111[2]

Received 18 August 1986/Accepted 18 March 1987

***Salmonella typhimurium*, enteroinvasive *Escherichia coli*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* possess the ability to enter intestinal epithelial cells. We used a quantitative tissue culture model employing HEp-2 cells to compare the abilities of these bacteria to enter epithelial cells. *S. typhimurium* and *Yersinia* species were highly infective for HEp-2 cells but were unable to replicate extensively intracellularly. Enteroinvasive *E. coli* exhibited low infectivity but replicated extensively intracellularly. The growth of enteroinvasive *E. coli* led to destruction of the HEp-2 monolayer, whereas *Yersinia* spp. and *S. typhimurium* were maintained intracellularly for prolonged periods without damage to the monolayer. The ability of enteroinvasive *E. coli* to enter HEp-2 cells required prior growth at 37°C; neither *S. typhimurium* nor *Yersinia* spp. exhibited this temperature dependence for cell entry. An *E. coli* K-12 derivative containing a 230-kilobase plasmid from enteroinvasive *E. coli* was constructed. This derivative shared all the invasive characteristics of the parental enteroinvasive strain, suggesting that determinants required for cell entry and intracellular multiplication were at least partially plasmid encoded. An HB101 derivative containing a cloned invasion determinant from *Y. pseudotuberculosis* was constructed in our laboratory. HEp-2 monolayers were coinfected with these two K-12 derivatives to compare invasion determinants from enteroinvasive *E. coli* with those of *Y. pseudotuberculosis* in a common genetic background. Results from these experiments suggest that these organisms reside within separate intracellular compartments.**

The genera *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia* all include species capable of causing an invasive diarrhea in humans. Despite a shared ability to enter intestinal epithelial cells, the pathology associated with infections by *Shigella* species or enteroinvasive *Escherichia coli* differs in several respects from that found in infections with *Salmonella* or *Yersinia* species. The pathology of shigellosis is striking in its localization. *Shigella* species and enteroinvasive *E. coli* produce an acute infection with considerable ulceration of the colon; the small bowel and local lymphatics are usually spared (6, 28, 29). Bacteremia is a rare event; indeed, infection does not usually spread beyond the lamina propria (28, 29, 34). In contrast, *Salmonella* and *Yersinia* species preferentially invade ilial tissue, although they frequently produce lesions throughout the bowel as well (4, 29, 36). Both are more likely to invade underlying tissues and enter the blood stream than are *Shigella* species. Infection with *Yersinia enterocolitica* or *Yersinia pseudotuberculosis* is frequently accompanied by mesenteric lymphadenitis and in many cases is associated with serious systemic disease (4).

Virulence is multifactoral in *Shigella*, *Yersinia*, and *Salmonella* species and requires both chromosomal (7, 12, 14, 19, 31, 35) and plasmid-mediated (3, 13, 16, 22, 26, 32, 39) traits. Characteristics thought to be involved in virulence include the possession of a particular lipopolysaccharide (16, 20, 24, 31, 39), the production of cytotoxin (11, 15, 23), and the ability to enter epithelial cells (6, 8, 9, 10, 19). The role these virulence determinants play in pathogenesis and the relationship between them is not fully understood, although the ability to enter epithelial cells is considered a prerequisite for virulence.

Formal and other investigators have reported that *Shigella* species replicate in the host (7–9), and investigations with cultured cells have shown that the ability to replicate intracellularly is correlated with the ability to escape from an endocytic vesicle (33). Work by several investigators has shown that *Salmonella typhimurium* and *Yersinia* species do not multiply intracellularly in cultured epithelial cells (5, 33), although they may do so in macrophages.

In *Shigella* species the expression of invasion determinants requires prior growth at 37°C (21). Genes requiring temperature-regulated expression have also been found in *Salmonella* species (17), *Yersinia pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (3, 11), although these genes have not been shown to play a role in invasion.

In this paper we report the results of studies comparing the abilities of enteroinvasive *E. coli*, *S. typhimurium*, *Y. enterocolitica*, and *Y. pseudotuberculosis* to enter and replicate within cultured epithelial cells. For these studies we used a modification of the HEp-2 cell invasion assay (38). We constructed *E. coli* K-12 derivatives which contain invasion genes from enteroinvasive *E. coli* and *Y. pseudotuberculosis* and used these to segregate invasion determinants from other genetic traits unique to *Yersinia* species or enteroinvasive *E. coli*. This allowed us to examine defined invasion determinants in a common chromosomal background.

* Corresponding author.
† Present address: Department of Biology, Middlebury College, Middlebury, VT 05753.

TABLE 1. Bacterial strains and plasmids

Strain	Relevant characteristics[a]	Plasmids (kb)	Source or reference
Escherichia coli II	Inv$^+$ Vir$^+$	230, small cryptic	Joy Miller[b]
Escherichia coli HB101	Str^r	None	1
Escherichia coli HB101(pSF204)	Inv$^+$ Str^r Amp^r	235	This work
Escherichia coli HB101::Tn*5*(pSF204)	Inv$^+$ Amp^r Kan^r	235	This work
Escherichia coli HB101(pRI203)	Inv$^+$ Amp^r Cam^r	9.4	14
Salmonella typhimurium 79–80	Inv$^+$ Vir$^+$	62	Sharon Abbott[c]
Yersinia enterocolitica 8081(pYV8081)	Inv$^+$ Vir$^+$	72	26
Yersinia enterocolitica 8081c	Inv$^+$	None	26
Yersinia pseudotuberculosis YPIII(pIB1)	Inv$^+$ Vir$^+$	66	3
Yersinia pseudotuberculosis YPIIIc	Inv$^+$	None	3

[a] Inv$^+$, Ability to invade HEp-2 cells; vir$^+$, virulence in animal model; Str, streptomycin; Amp, ampicillin; Kan, kanamycin; Cam, chloramphenicol.
[b] Centers for Disease Control, Atlanta, Ga.
[c] California State Department of Health, Berkeley.

MATERIALS AND METHODS

Bacterial strains. Strains used are listed in Table 1.

Media. HEp-2 cells were maintained in RPMI 1640 medium (Irvine Biochemical) supplemented with 5 mM glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 5% fetal calf serum (GIBCO). Bacteria were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates, on minimal medium supplemented with 1 μg of thiamine per ml and 0.2% glucose, or in brain heart infusion broth. Antibiotics were added in the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 25 μg/ml.

Construction of HB101 derivatives. A 230-kilobase plasmid from a clinical isolate of enteroinvasive *E. coli*, *E. coli* 11 (O:124) was genetically marked with the ampicillin resistance transposon, Tn*801*, by using the plasmid pMR5 (27). The virulence plasmid containing the transposon Tn*801* was then mobilized into *E. coli* K-12 HB101 by using F′ *lac* ts:Tn*10*. The mobilizing plasmid was lost at a high frequency when grown at 37°C in the absence of tetracycline. One derivative, HB101(pSF204), was utilized for further work. Kanamycin-resistant (Kan^r) derivatives of HB101(pSF04) were obtained by infection of HB101(pSF204) with lambda *b*221 *c*1857::Tn*5* *O*am23 *P*am80 (2, 30). Kanamycin-resistant organisms were screened for the ability to infect HEp-2 cells, and plasmid digests were undertaken to determine the location of Tn*5* insertions. One derivative, HB101:: Tn*5*(pSF204), which retained the ability to invade HEp-2 cells and did not contain a Tn*5* insertion in the virulence plasmid pSF204, was used for coinfection experiments.

HB101(pR1203) is an HB101 derivative constructed by Isberg and Falkow (14). It contains a plasmid with a 3.1-kilobase insert cloned from the chromosome of *Y. pseudotuberculosis* into plasmid vector pBR325. This clone confers on HB101 the ability to enter HEp-2 cells. It also carries plasmid-mediated drug markers for chloramphenicol and tetracycline.

Determination of efficiency of bacterial entry into HEp-2 cells. HEp-2 cells were cultured on glass cover slips (Bellco Glass, Inc., Vineland, N.J.) placed in the bottom of 1-dram (3.7-ml) glass shell vials (American Scientific Products, McGaw Park, Ill.) or directly on the bottom of glass vials (38). Bacteria were grown overnight at 37°C on Trypticase soy agar plates. On the following day 3 ml of brain heart infusion broth was inoculated with these organisms, and they were grown with shaking at 37°C for 2 h. This 2-h growth was important for enteroinvasive *E. coli* because of the fact that bacteria undergoing logarithmic growth enter epithelial cells more efficiently than do bacteria in the stationary growth phase. Bacteria were washed in phosphate-buffered saline (pH 7.4) and resuspended in RPMI 1640 medium at a density of 3×10^7 organisms per ml. A 1-ml sample of this suspension was added to each monolayer, which resulted in a ratio of 100 bacteria per HEp-2 cell. For *Y. pseudotuberculosis* and *Y. enterocolitica* containing the *Yersinia* virulence plasmids, a lower inoculum, 4×10^6/ml, was used to avoid detachment of the monolayer caused by cytotoxicity (11, 25). The bacterial inoculum was centrifuged onto the HEp-2 monolayer at $800 \times g$ for 10 min as described previously (38). Infected monolayers were incubated for 2 h in 5% CO_2 at 37°C, washed three times with phosphate-buffered saline (pH 7.4), and covered with RPMI 1640 medium containing gentamicin (100 μg/ml) (37). After 1 h the monolayer was washed six times with phosphate-buffered saline and flooded with 1% Triton X-100 for 5 min to release intracellular bacteria. Dilutions were made of the lysed monolayer and plated on Trypticase soy agar plates containing appropriate antibiotics. Experiments were run in triplicate and repeated at least five times per isolate.

Intracellular multiplication. HEp-2 monolayers were cultured in glass vials and infected as described above. After a 2-h infection, RPMI 1640 medium containing gentamicin (50 μg/ml) was added to the monolayer to kill extracellular bacteria. For virulent isolates of *Y. enterocolitica* and *Y. pseudotuberculosis*, the infection period was shortened to 1 h to prevent detachment of the monolayer. The number of CFU per vial was determined for 0-, 2-, 4-, and 6-h time points from the end of the 2-h infection period. The number of CFU present at 2-, 4-, and 6-h time points was determined and compared with the number of CFU present at the first time point (0 h) to assess the relative increase in intracellular bacteria. We did not extend the experiment beyond the 6-h time point because infection with enteroinvasive *E. coli* 11 and HB101(pSF204) led to destruction of the HEp-2 monolayer at this time. Infected monolayers grown on cover slips were fixed with methanol and stained with Giemsa stain after 6 and 24 h of incubation to evaluate the integrity of the monolayer. Experiments were run in duplicate and repeated at least six times per strain.

Temperature dependence. Duplicate cultures of isolates to be tested were grown overnight at 30 and 37°C. The following morning, tubes containing brain heart infusion broth were inoculated with these cultures and grown for an additional 2 h with shaking at 30 and 37°C. HEp-2 monolayers were infected for 2 h as described above, and incubation was

continued for an additional 2 h in the presence of fresh RPMI 1640 medium containing gentamicin (100 μg/ml). Infected monolayers were washed, lysed, and plated to determine the number of bacteria which had survived gentamicin killing. Experiments were done in triplicate and repeated at least five times per isolate.

Coinfection with HB101::Tn*5*(pSF204) and HB101(pRI203). Vials containing HEp-2 monolayers were each coinfected with a mixture of 3×10^7 cells of a Kan^r HB101 derivative containing the enteroinvasive *E. coli* plasmid pSF204 per ml and chloramphenicol-resistant (Cam^r) cells (3×10^6/ml) of an HB101 derivative containing a *Yersinia* invasion gene, pRI203. After a 3-h infection, fresh medium containing gentamicin (50 μg/ml) was added to washed monolayers to kill extracellular bacteria. HEp-2 cells were lysed with Triton X-100 at 0, 2, 4, and 6 h from the end of the infection period. Lysates were spread on Trypticase soy agar plates containing kanamycin (20 μg/ml) and chloramphenicol (20 μg/ml). The ratio of HB101::Tn*5*(pSF204) to HB101(pRI203) at 0, 2, 4, and 6 h after the infection period was calculated. Ten times fewer HB101 cells carrying the *Yersinia* invasion gene were added than HB101 cells carrying enteroinvasive *E. coli* invasion genes to compensate to some extent for the more efficient invasion of *Yersinia* species. Experiments were run in duplicate and repeated at least five times.

Conjugation and mobilization. Strains were grown to the early log phase, and plate matings were performed. Matings were done at 30°C to stabilize the mobilizing plasmid F′ *lac* ts:Tn*10*.

RESULTS

Efficiency of entry. HEp-2 monolayers were infected with *S. typhimurium* 79-80, *Y. enterocolitica* 8081(pYV8081), *Y. pseudotuberculosis* III(pIB1), and enteroinvasive *E. coli* 11 to compare the ability of these strains to enter HEp-2 cells. *E. coli* K-12 HB101 was used as a negative control. The number of bacteria recovered after a 2-h infection and 1 h of gentamicin treatment was compared with the number of bacteria initially added to the monolayer to determine the efficiency with which bacteria became internalized within HEp-2 cells. Efficiency was expressed as the percentage of CFU recovered from the lysed infected monolayer (after gentamicin killing) compared with the original inoculum. Data shown in Table 2 represent the mean and standard deviation of one experiment. *S. typhimurium* 79-80, *Y. enterocolitca* 8081(pYV8081), and *Y. pseudotuberculosis* III(pIB1) invaded HEp-2 cells more efficiently than did enteroinvasive *E. coli* 11. Varying the number of bacteria added per HEp-2 cell from 6 to 666 affected the percentage of the inoculum internalized. For *Yersinia* species in particular there was a marked decrease in the percentage of bacteria internalized as the inoculum was increased, but in all cases the infectivity of enteroinvasive *E. coli* 11 was at least threefold lower than that of *Yersinia* species. Some intracellular multiplication may have occurred by the end of the infection and gentamicin killing period. However, an infection period of 2 h was necessary for optimal invasion by enteroinvasive *E. coli* and *S. typhimurium*. Observations of Giemsa-stained slides were consistent with the data presented in Table 2. In infections with *Yersinia* species, every HEp-2 cell appeared to contain numerous bacteria; in infections with *S. typhimurium*, typically 85 to 90% of the HEp-2 cells appeared to contain one or more bacteria; whereas in infections with enteroinvasive *E. coli*, 60 to 70% of the HEp-2 cells appeared to contain intracellular bacteria (data not shown).

TABLE 2. Efficiency of invasion

Strain	% Inoculum protected from gentamicin killing after 2-h infection period[a]
E. coli HB101	0.003 ± 0.0
Enteroinvasive *E. coli* II	2.9 ± 0.23
S. typhimurium 79–80	16.3 ± 6.5
Y. enterocolitica 8081(pYV8081)	21.0 ± 1.7
Y. pseudotuberculosis III(pIB1)	11.0 ± 4.1
E. coli HB101(pSF204)	1.6 ± 0.9
E. coli HB101(pRI203)	23.0 ± 3.3

[a] Inoculum = 3×10^7 cells. Mean plus standard deviation of triplicate samples from a typical experiment.

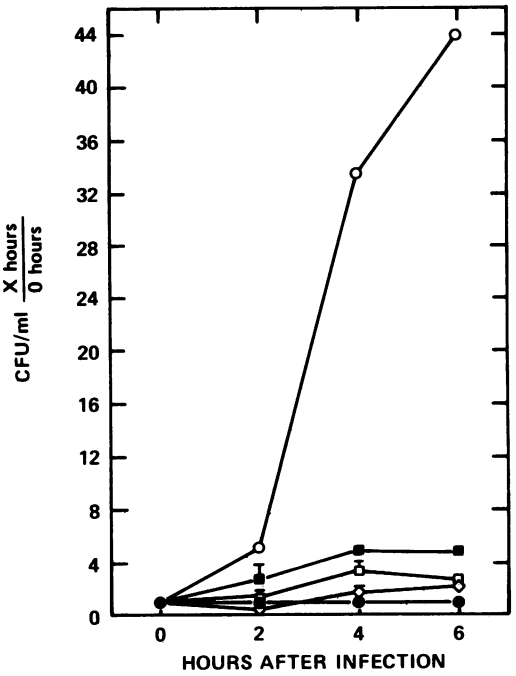

FIG. 1. Kinetics of bacterial growth within HEp-2 cells. Symbols: ●, HB101; ◇, *S. typhimurium* 79-80; ■, *Y. enterocolitica*(pYV8081); □, *Y. pseudotuberculosis*(pIB1); ○, enteroinvasive *E. coli*. Each point represents the mean of duplicate samples. Data shown are from one of six experiments.

Kinetics of intracellular multiplication. To determine whether isolates tested were capable of intracellular multiplication, we compared the number of CFU of bacteria protected from gentamicin killing at 2, 4, and 6 h after infection with the number of CFU of bacteria present after the initial 2-h infection period. Experiments were run in duplicate and repeated five times per isolate. The results of a typical experiment are shown in Fig. 1. Since extracellular bacteria were killed with gentamicin, an increase in the CFU of bacteria recovered over the time of the study was taken as a reflection of intracellular growth. There was initially a slight increase in *S. typhimurium*, *Y. enterocolitica*, and *Y. pseudotuberculosis* between the 2- and 4-h time points. This may represent bacteria which were in the process of dividing at the time of cell entry, partial protection from gentamicin killing of bacteria bound to the HEp-2 cell, or a limited degree of intracellular multiplication. However, after 4 h there was no further increase in viable counts recovered

TABLE 3. Effect of growth temperature on invasion

Strain	CFU/ml[a] recovered after gentamicin killing at a growth temp of:	
	30°C	37°C
E. coli HB101	$(2.5 \pm 1.4) \times 10^3$	$(1.0 \pm 0.0) \times 10^3$
S. typhimurium 79–80	$(5.2 \pm 2.1) \times 10^5$	$(2.0 \pm 0.8) \times 10^6$
E. coli HB101(pSF204)	$(1.0 \pm 0.0) \times 10^3$	$(2.3 \pm 0.47) \times 10^5$
E. coli II	$(2.6 \pm 0.47) \times 10^3$	$(2.3 \pm 0.47) \times 10^5$
Y. enterocolitica 8081(pYV8081)	$(8.0 \pm 0.81) \times 10^6$	$(3.0 \pm 0.82) \times 10^6$
Y. enterocolitica 8081c	$(1.3 \pm 0.47) \times 10^7$	$(3.0 \pm 0.82) \times 10^6$
Y. pseudotuberculosis III(pIB1)	$(5.7 \pm 1.7) \times 10^4$	—[b]
E. coli HB101(pRI203)	$(1.6 \pm 0.47) \times 10^6$	$(1.0 \pm 0.0) \times 10^6$
Y. pseudotuberculosis IIIc	$(3.7 \pm 0.94) \times 10^5$	$(2.3 \pm 0.47) \times 10^4$

[a] Numbers represent mean and standard deviation of triplicate samples from a typical experiment.
[b] —, Loss of monolayer prevented accurate determination.

from *S. typhimurium*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. This was true even after 48 h of incubation (data not shown). In contrast to these findings, larger numbers of enteroinvasive *E. coli* were recovered at each successive time point.

Temperature dependence. Results from our experiments comparing the ability of *S. typhimurium*, *Y. enterocolitica*, *Y. pseudotuberculosis*, and enteroinvasive *E. coli* grown at 30 and 37°C to enter HEp-2 cells are shown in Table 3. Enteroinvasive *E. coli* required prior growth at 37°C to express the invasive phenotype, whereas *S. typhimurium*, *Y. enterocolitica*, and *Y. pseudotuberculosis* were clearly able to invade HEp-2 cells when grown at 30 or 37°C. We consistently recovered lower numbers of bacteria from plasmid-containing *Yersinia* species compared with plasmidless derivatives. Whether this reflected lower expression of invasion genes in plasmid-containing strains or increased monolayer destruction owing to plasmid-encoded cytotoxin is not certain. The cytotoxicity of plasmid-containing *Yersinia* species made it difficult to wash monolayers infected with virulent *Y. enterocolitica* or *Y. pseudotuberculosis* without loosening some HEp-2 cells. This loss of cells would also result in lower recovery of intracellular bacteria. It was not possible to maintain an intact monolayer in assays with plasmid-containing isolates of *Y. pseudotuberculosis* grown at 37°C because of cytotoxin activity.

Interaction of defined *E. coli* K-12 derivatives carrying invasion determinants from enteroinvasive *E. coli* or *Y. pseudotuberculosis* with HEp-2 cells. The invasive ability of *E. coli* HB101 carrying plasmid-mediated invasion determinants from enteroinvasive *E. coli* [HB101(pSF204)] was compared with that of the parental enteroinvasive *E. coli* 11. Similarly, the invasive ability of *E. coli* HB101 carrying *Yersinia* invasion determinants [HB101(pRI203)] was compared with that of the parental *Y. pseudotuberculosis* strain. These results are shown in Fig. 2A and B. We found that HB101(pSF204) entered HEp-2 cells and replicated intracellularly at levels comparable to, albeit somewhat lower than, those of the parental enteroinvasive *E. coli* strain. Further, infection of HEp-2 cells with HB101 (pSF204) for over 6 h led to the destruction of the monolayer. The expression of the invasive phenotype in HB101 (pSF204) required prior growth at 37°C. Like the parental enteroinvasive *E. coli* 11, HB101(pSF204) was essentially noninvasive when grown overnight at 30°C (Table 3). In

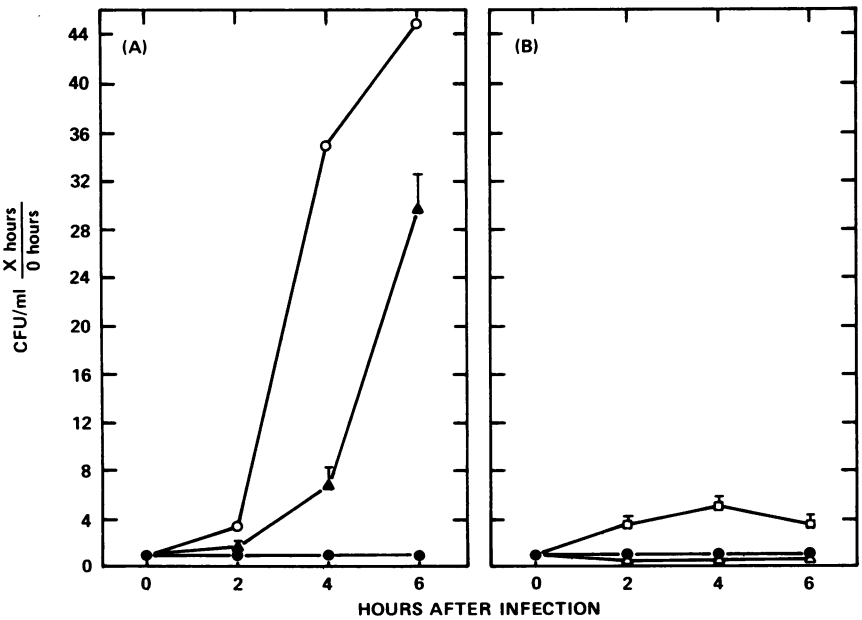

FIG. 2. (A) HEp-2 lysis assay for intracellular multiplication. Comparison of an HB101 derivative containing the virulence plasmid pSF204 with the parental strain, enteroinvasive *E. coli* 11, in terms of the ability to enter and replicate within HEp-2 cells. Symbols: ○, enteroinvasive *E. coli* 11; ▲, HB101(pSF204); ●, HB101. Points represent mean of duplicate samples from one of five experiments. (B) HEp-2 lysis assay for intracellular multiplication. Comparison of an HB101 derivative containing an invasion gene from *Y. pseudotuberculosis* III [HB101(pRI203)] with the parental strain, *Y. pseudotuberculosis* III, in terms of the ability to enter and replicate within HEp-2 cells. Symbols: □, *Y. pseudotuberculosis* IIIc; △, HB101(pRI203); ●, HB101. Points represent mean of duplicate samples. Data are from one of five experiments.

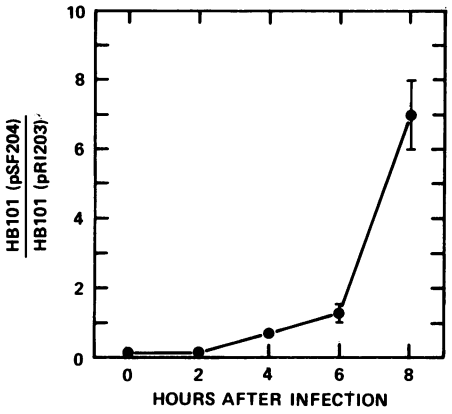

FIG. 3. HEp-2 lysis assay for intracellular multiplication: coinfection experiment. Kinetics of intracellular growth of HB101 derivatives containing invasion genes from enteroinvasive *E. coli* 11 [HB101(pSF204)] and *Y. pseudotuberculosis* III [HB101(pRI203)] when used to coinfect HEp-2 cells. Points represent mean of duplicate samples from a typical experiment.

short, infection of the HEp-2 monolayer with HB101 (pSF204) paralleled that of infection with enteroinvasive *E. coli* 11 in every respect.

Infection of HEp-2 cells with the HB101 derivative carrying *Yersinia* invasion determinations [HB101(pRI203)] similarly paralleled our results with parental strain *Y. pseudotuberculosis* III. HB101(pRI203) invaded HEp-2 cells with an efficiency comparable to that of *Y. pseudotuberculosis* and like the parental strain did not multiply intracellularly or lead to destruction of the monolayer. There was little difference, however, in CFU recovered from HEp-2 cells infected with bacteria grown at 30 or 37°C (Table 3). These results led us to investigate how HB101(pSF204) and HB101(pRI203) would interact in a mixed infection.

Coinfection with HB101::Tn*5*(pSF204) and HB101(pRI203). When monolayers were coinfected with HB101::Tn*5* (pSF204) and HB101(pRI203), we found that the ratio of HB101::Tn*5*(pSF204) to HB101(pRI203) increased over the period assayed (Fig. 3). Initially, the ratio of HB101::Tn*5* (pSF204) to HB101(pRI203) was only 0.15. This reflects the lower infectivity of HB101(pSF204) for HEp-2 cells compared with that of HB101(pRI203) and parallels the results found with parental strains (Table 2). The recovery of intracellular bacteria at 2, 4, and 6 h after infection showed an increase in the ratio of HB101::Tn*5*(pSF204) to HB101(pRI203) at each successive time point (Fig. 3).

DISCUSSION

We found a number of differences in the invasive phenotypes of *Salmonella* species, *Yersinia* species, and enteroinvasive *E. coli*. Differences in the level of infectivity and the ability to multiply intracellularly, as well as differences in expression based on growth temperature, suggest that each of these organisms has evolved a unique strategy for entry into and survival within intestinal epithelial cells. Experiments with HB101 derivatives emphasize the fact that plasmid-encoded determinants, in the case of enteroinvasive *E. coli* 11, and a single chromosomal gene, in the case of *Y. pseudotuberculosis*, are sufficient to produce the invasive phenotype found in parental strains.

The fact that *S. typhimurium* and *Yersinia* species do not require prior growth at 37°C to express invasion determinants is consistent with their epidemiology since both are often food- or waterborne pathogens. Although little is known concerning the epidemiology of enteroinvasive *E. coli*, the fact that prior growth at 37°C is required for expression of invasion suggests that its mode of transmission is from host to host, as is often the case with *Shigella* species.

Results from our coinfection experiment are open to several interpretations. One possibility is that the two strains may enter separate populations of HEp-2 cells. Considering the high infectivity of *Yersinia* species for epithelial cells, this seems unlikely. A more reasonable explanation is that the two populations are present in different compartments within the same cell. This is consistent with the observed differences between the two in intracellular multiplication. If both bacterial populations were in the same compartment, it is difficult to see why both would not replicate intracellularly since both would presumably have access to the host cell cytoplasm.

Much remains to be known about the process by which bacteria gain entry to epithelial cells. It is tempting to postulate that bacteria induce their own uptake via receptor-mediated endocytosis. The considerable differences between the invasive phenotypes of *Salmonella* species, *Yersinia* species, and enteroinvasive *E. coli*, however, allow for the possibility that the process of entry is not the same for all these pathogens. A good deal of work remains to be done in this area.

Finally, we would like to emphasize that these comparative studies of invasion have focused on bacterial entry into one cell type only, a single line of cultured epithelial cells. It is probable that HEp-2 cells and native intestinal epithelial cells differ in many surface structures. These differences could include differences in receptors, or in the density of receptors important for bacterial entry.

In addition, for *Yersinia* and *Salmonella* species, which may cause disseminated infection in the host, invasion of the epithelial cell may be only an initial, or even possibly an incidental, event in pathogenesis. The epithelial cell may be nothing more than a safe corridor for these organisms to pass through on their way to deeper tissue where they are picked up by macrophages. To understand virulence in *Salmonella* or *Yersinia* species, it will be important to study the interactions of these organisms with macrophages as well as with epithelial cells.

ACKNOWLEDGMENTS

This work was supported by U.S. Army Medical Research and Development Command DAMD 17-85-C-5163 and by subcontract UC25959 from the University of California. P.S. was a Johnson & Johnson predoctoral fellow. R.I. was a Jane Coffin Child postdoctoral fellow.

LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. **Berg, D. E.** 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn*5*, p. 205–212. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Bolin, I., L. Norlander, and H. Wolf-watz.** 1982. Temperature-inducible outer membrane proteins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are associated with the virulence plasmid. Infect. Immun. **37**:506–512.
4. **Bradford, W. D., P. S. Noce, and L. T. Gutman.** 1974. Pathologic features of enteric infection with *Yersinia enteroco-*

litica. Arch. Pathol. **98**:17–22.
5. **Devenish, J. A., and D. A. Schiemann.** 1981. HeLa cell infection by *Yersinia enterocolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect. Immun. **32**:48–55.
6. **Dupont, H. L., S. B. Formal, R. B. Hornick, M. Snyder, J. P. Libonati, D. G. Sheahan, E. H. La Brec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli*. N. Engl. J. Med. **285**:1–9.
7. **Formal, S. B., E. H. La Brec, T. H. Kent, and S. Falkow.** 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. **89**:1374–1382.
8. **Gemski, P., and S. B. Formal.** 1975. Shigellosis: an invasive infection of the gastrointestinal tract, p. 165–169. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
9. **Gemski, P., Jr., A. Takeuchi, O. Washington, and S. B. Formal.** 1972. Shigellosis due to *Shigella dysentariae*. I. Relative importance of mucosal invasion versus toxin production in pathogenesis. J. Infect. Dis. **5**:523–530.
10. **Giannella, R. A., O. Washington, P. Gemski, and S. B. Formal.** 1973. Invasion of HeLa cells by *S. typhimurium*: a study of invasiveness of *Salmonella*. J. Infect. Dis. **128**:69–75.
11. **Goguen, J., W. S. Walker, T. P. Hatch, and J. Yother.** 1986. Plasmid-determined cytotoxicity in *Yersinia pestis* and *Yersinia pseudotuberculosis*. Infect. Immun. **51**:788–794.
12. **Heesemann, J., B. Algermissin, and R. Lambs.** 1984. Genetically manipulated virulence of *Yersinia enterocolitica*. Infect. Immun. **46**:105–110.
13. **Helmuth, R., R. Stephan, C. Bunge, B. Hoog, A. Steinbeck, and E. Bulling.** 1985. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. Infect. Immun. **48**:175–182.
14. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317**:262–264.
15. **Jacewicz, M., and G. T. Keusch.** 1983. Pathogenesis of Shigella diarrhea. VIII. Evidence for a translocation step in the cytotoxic action of Shiga toxin. J. Infect. Dis. **148**:844–854.
16. **Jones, G. W., D. K. Rabert, D. M. Svinarich, and H. J. Whitfield.** 1982. Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megaldalton plasmids. Infect. Immun. **38**:476–486.
17. **Jones, G. W., and L. A. Richardson.** 1981. The attachment to and invasion of HeLa cells by *Salmonella typhimurium*: the contribution of mannose-sensitive, mannose-resistant hemagglutinating activities. J. Gen. Microbiol. **127**:361–370.
18. **Kopecko, D. J., O. Washington, and S. B. Formal.** 1980. Genetic and physical evidence for plasmid control of *Shigella sonnei* form I cell surface antigen. Infect. Immun. **29**:207–214.
19. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23**:1714–1722.
20. **Makela, P. H., V. V. Valtonen, and M. Voltonen.** 1973. The role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella*. J. Infect. Dis. **128**:81–85.
21. **Maurelli, A., B. Blackmon, and R. Curtiss III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195–201.
22. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. **43**:397–401.
23. **O'Brien, A. D., J. W. Peterson, and S. B. Formal.** 1984. Enterotoxic diseases due to shigella and salmonella, p. 3–74. *In* M. Hardegree, W. H. Hobig, and A. T. Ta (ed.), Handbooks of natural toxins. II. Bacterial toxins. Marcel Dekker, Inc., New York.
24. **Pedersen, K. B., S. Winblad, and V. Birsch.** 1979. Studies of the interaction between different O-serotypes of *Yersinia enterocolitica* and HeLa cells. Acta Pathol. Microbiol. Scand. Sect. B **87**:141–145.
25. **Portnoy, D. A., S. L. Mosely, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**:775–782.
26. **Portnoy, D. A., H. F. Bland, P. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
27. **Robinson, M. K., P. M. Bennet, S. Falkow, and H. M. Dodd.** 1980. Isolation of a temperature-sensitive derivative of RP1. Plasmid **3**:343–347.
28. **Rout, W. R., S. B. Formal, and R. A. Giannella.** 1975. Pathophysiology of *Shigella* diarrhea in the rhesus monkey: intestinal transport, morphological and bacteriological studies. Gastroenterology **67**:59–70.
29. **Rutgeerts, P., K. Geboes, E. Ponette, G. Caremans, and G. Vantroppen.** 1982. Acute infective colitis caused by endemic pathogens in Western Europe. Endoscopic features. Endoscopy **14**:212–219.
30. **Ruvkin, G. B., and F. M. Ausubel.** 1981. A general method for site directed mutagenesis in prokaryotes. Nature (London) **289**:85–88.
31. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, and S. B. Formal.** 1983. Alterations in the pathogenicity of *Escherichia coli* K-12 following the transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect. Immun. **39**:1392–1402.
32. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34**:75–83.
33. **Sansonetti, P. J., A. Ryter, P. Clerc, A. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461–469.
34. **Takeuchi, A., S. B. Formal, and H. Sprinz.** 1958. Experimental acute colitis in the rhesus monkey following peroral infection with *Shigella flexneri*. Am. J. Pathol. **52**:503–529.
35. **Timmis, K. N., C. L. Clayton, and T. Sekizaki.** 1985. Localization of Shiga toxin gene in the region of *Shigella dysentaeriae* 1 chromosome specifying virulence functions. FEMS Microbiol. Lett. **30**:301–305.
36. **Vantrappen, G., H. O. Agg, K. Geboes, and E. Ponette.** 1982. *Yersinia* enteriditis. Med. Clin. North Am. **66**:639–653.
37. **Vaudaux, P., and F. A. Waldvogel.** 1979. Gentamicin antibacterial activity in the presence of human PMN's. Antimicrob. Agents Chemother. **16**:743–749.
38. **Vesikari, T., J. Bromirska, and M. Maki.** 1982. Enhancement of invasiveness of *Yersinia enterocolitica* and *Escherichia coli* in Hep-2 cells by centrifugation. Infect. Immun. **36**:834–836.
39. **Watanabe, H., and K. N. Timmis.** 1984. A small plasmid in *Shigella dysenteriae* 1 specifies one or more functions for O antigen production and bacterial virulence. Infect. Immun. **43**:391–396.