Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6473–6477, July 1993
Microbiology

Yersinia enterocolitica invasin: A primary role in the initiation of infection

(invasion/*inv* mutant/pathogenesis)

JEFFREY C. PEPE* AND VIRGINIA L. MILLER*†

*Department of Microbiology and Molecular Genetics and †the Molecular Biology Institute, University of California, Los Angeles, CA 90024-1489

Communicated by Stanley Falkow, March 8, 1993

ABSTRACT **The ability to invade the intestinal epithelium of mammals is an essential virulence determinant of *Yersinia enterocolitica*. The chromosomally encoded *Y. enterocolitica* 8081v invasion gene, *inv*, was disrupted to assess its role in pathogenesis. The *inv* mutant (JP273v) was ≈80-fold less invasive than wild type for cultured epithelial cells. When mice were infected intragastrically, up to 10^7 fewer JP273v were recovered from Peyer's patches early (6–18 hr) after infection compared with wild type. Analysis of the course of infection revealed that the *inv* mutant had distinct differences relative to wild type in the distribution of visible infectious foci and in tissue colonization; however, the mutant and wild-type strains had similar LD_{50} values for both orally and intraperitoneally infected mice. The invasion defect of the *inv* mutant was fully complemented *in vitro* and *in vivo* by introduction of the wild-type *inv* gene in trans. The *inv* gene product, invasin, appears to play a vital role in promoting entry during the initial stage of infection. During the subsequent establishment of a systemic infection, invasin may be of secondary importance, since the *Y. enterocolitica inv* mutant was as proficient as wild type at causing a fatal infection in mice. Based on these data, we discuss the role of invasin in a naturally occurring *Y. enterocolitica* infection.**

The three pathogenic species of the genus *Yersinia* invade host tissues and cause disease syndromes ranging from gastroenteritis (*Y. enterocolitica* and *Y. pseudotuberculosis*) to plague (*Y. pestis*). *Yersinia* pathogenesis is multifactorial in that chromosomal and plasmid genes act in concert to produce infection and disease. All pathogenic *Yersinia* species harbor a related virulence plasmid that encodes several thermally regulated virulence determinants (1). Cellular penetration, a phenotype that can be plasmid- or chromosomally encoded, permits bacteria to escape initial host defenses and provides them with an opportunity to spread to other tissues within the host.

Early studies showed that pathogenic strains of *Y. enterocolitica* could invade eukaryotic cells *in vitro* whereas nonpathogenic strains could not (2). A molecular analysis of cellular penetration led to the cloning of the chromosomally encoded invasion gene (*inv*) of *Y. pseudotuberculosis* (3). Invasin, the *inv* gene product, initiates the entry process directly by attaching to mammalian cell receptors, which include multiple members of the integrin superfamily (4). Subsequently, the homologous *inv* gene from *Y. enterocolitica*, as well as a second chromosomal invasion gene, *ail*, were cloned (5). A third invasion factor, encoded by the virulence plasmid and referred to as P.D.E., promotes low-level invasion of tissue culture cells. P.D.E. is detectable only in *inv* mutants of *Y. enterocolitica* and *Y. pseudotuberculosis* (unpublished data and ref. 6). Although several invasion factors exist, the highest level of penetration into cultured cells is promoted by invasin; thus, we focused on the role of invasin during a *Y. enterocolitica* infection.

Infection with *Y. enterocolitica* generally occurs when a susceptible host ingests contaminated food or water, whereupon bacteria migrate to the terminal ileum. Here the organisms most likely adhere to the M cells that overlay the lymphoid follicles [Peyer's patches (PP)] lining the gastrointestinal tract, and primarily penetrate through these cells to colonize the PP (7, 8). It was previously reported that a *Y. pseudotuberculosis inv* mutant is unaffected in its LD_{50} for mice (9). The studies reported here were initiated to determine whether this was also the case for a *Y. enterocolitica inv* mutant and to examine the infectious process in more detail. We found that a *Y. enterocolitica inv* null mutant was defective in its ability to invade tissue culture cells and in its ability to efficiently penetrate the intestinal epithelium. Furthermore, the *inv* mutant and the wild-type strain showed distinctly different kinetics of infection.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Plasmid Constructs. Wild-type strain 8081v (Nal^r) of *Y. enterocolitica*, obtained from R. Martinez (University of California, Los Angeles), was used as the parent strain to construct the *inv* mutant JP273v (described below). The "v" designation refers to the presence of the *Y. enterocolitica* virulence plasmid pYV8081. *Escherichia coli* SM10λ*pir* (10) was used to conjugally transfer all plasmids to *Y. enterocolitica*. Bacteria were grown in Luria broth (LB) and minimal medium M63 (11). The following concentrations of antibiotics were used when appropriate: naladixic acid (Nal), 30 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 40 μg/ml.

The mobilizable suicide vector pINT192 was constructed as follows. The 2700-bp *Nhe* I–*Sal* I fragment from pJM703.1 (12), which contains *ori*R6K and *mob*RP4, was ligated to the 2700-bp *Nhe* I–*Sal* I fragment from pACYC184 (13) that encodes chloramphenicol acetyltransferase. An 1100-bp *Hin*cII fragment was deleted and replaced with a unique *Bgl* II site; this plasmid is referred to as pEP184 and will only replicate in a pir^+ strain. Plasmid pVM112.1 was generated from pVM112 (14) by converting the *Nde* I site within the *inv* coding region into a *Bgl* II site. The 1400-bp *Bgl* II fragment internal to the *inv* coding region from pVM112.1 was subcloned into pEP184 to generate pINT192.

The low-copy, mobilizable *inv* complementation plasmid pEP301 was constructed as follows. The 1800-bp *Bam*HI fragment containing *mob*RP4 derived from pJM703.1 was subcloned into pCP178 (C. Pepe, unpublished plasmid), a low-copy (pSC101 derivative) plasmid that encodes chloramphenicol acetyltransferase, to generate pMW1823. The 1400-bp *Pst* I kanamycin-resistance GenBlock (Pharmacia)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: cfu, colony-forming unit(s); PP, Peyer's patch(es); MLN, mesenteric lymph node(s).

was subcloned into pMW1823 to generate pEP1823. The 3700-bp *Eco*RI fragment containing the entire *inv* coding sequence, flanked by ≈500 bp upstream and downstream that lack any significant open reading frames, was subcloned into pEP1823 to generate the *inv*-complementing vector pEP301.

Generation and Characterization of a *Y. enterocolitica inv* Mutant. Filter matings of the *E. coli* SM10λ*pir*(pINT192) donor with the *Y. enterocolitica* 8081v recipient were performed as described (10). Integration of pINT192 by homologous recombination at the *inv* locus results in duplicate, nonidentical, truncated copies of the *inv* gene separated by the suicide vector. The *inv* mutant JP273v was verified by Southern hybridization with several restriction enzymes (15) and by Western blot analysis with rabbit polyclonal anti-invasin (unpublished results; ref. 14) as described.

Tissue Culture Invasion Assay. Bacterial cultures were grown for 16 hr at 23°C or 37°C and then added to subconfluent HEp-2 human epithelial cell monolayers at a multiplicity of infection of ≈100. The rest of the invasion assay was performed as described (5). Results are expressed as % invasion = 100 × (no. of bacteria resistant to gentamicin/initial no. of bacteria).

Animal Experiments. Bacteria were grown with aeration for 18 hr at 23°C, washed with phosphate-buffered saline (PBS), and diluted to the appropriate infectious dose in PBS. Virus-free 6- to 7-week-old female BALB/c mice were infected as described below. Mice surviving infection for the length of an experiment were killed by cervical dislocation before being dissected. Organs harvested from surviving or fatally infected mice were weighed, suspended in PBS as described below, and homogenized in a Stomacher (Tekmar, Cincinnati). Homogenates were diluted and spread-plated to quantitate the colony-forming units (cfu) recovered from each organ. Comparison of the efficiency of plating on control LB/Nal plates versus LB/Nal plates supplemented with the appropriate antibiotic(s) was used to determine whether the plasmids and the chromosomal *inv* mutation were maintained after passage through mice. Randomly selected colonies from the control LB/Nal plates were examined by Southern blot analysis for retention of the plasmids, the *inv* mutation, or both. The four types of animal assays performed are described below. Each experiment was done at least three times; data presented from single experiments are representative of all experiments performed.

(i) In vivo *invasion*. Three groups of four mice were infected intragastrically with ≈10^8 bacteria in 0.25 ml via a 21-gauge feeding tube. Four mice were killed by cervical dislocation at 6, 18, and 32 hr postinfection. The four PP proximal to the ileal–cecal junction were excised from each mouse and suspended in 0.5 ml of PBS. The limit of detection was ≈300 cfu/g of tissue.

(ii) Oral LD_{50}. Five groups of six mice were infected intragastrically with successive 10-fold dilutions of a bacterial suspension (10^4–10^8 bacteria). The mice were monitored twice daily for 14 days. The liver, spleen, and PP were harvested from the mice that died and from mice that survived the length of the experiment. Each organ was suspended in 10 ml of PBS. The limit of detection was 300, 800, and 1500 cfu/g of liver, spleen, and PP, respectively.

(iii) Kinetics of infection. Mice were infected intragastrically with 1 LD_{50} (5×10^6 bacteria) and divided into six groups of six mice each that corresponded to the day of sacrifice (1, 2, 4, 7, 10, or 14 days postinfection). Four mice from each group were analyzed unless fewer than four mice survived to the day of sacrifice. Mice that died during the course of the experiment were also analyzed (see Table 2). The PP, mesenteric lymph nodes (MLN), spleen, and liver were harvested from each mouse and suspended in 0.5, 0.5, 3, and 5 ml of PBS, respectively. The limit of detection was 300, 200, 350, and 60 cfu/g of tissue, respectively.

(iv) i.p. LD_{50}. Five groups of six mice each were injected intraperitoneally with 0.2 ml via a 23-gauge needle with successive 10-fold dilutions of a bacterial suspension (10–10^5 bacteria). The mice were monitored twice daily for 14 days. Spleens were harvested from all mice. Each spleen was suspended in 10 ml of PBS. The limit of detection was 800 cfu/g of tissue.

RESULTS

***In Vitro* Invasion by the *inv* Mutant.** The *Y. enterocolitica inv* mutant JP273v grown at 23°C was down 80-fold for invasion compared with 8081v (Table 1). When the *inv*-complementing plasmid pEP301 was introduced into JP273v, the invasive phenotype was restored (Table 1), showing that the invasion-defective phenotype of JP273v was due to the disruption of *inv* and not to polar effects on downstream genes. JP273v remained defective for invasion when harboring the vector pEP1823 alone (Table 1). Invasion of cultured epithelial (HEp-2) cells by *Y. enterocolitica* 8081v showed thermoregulation; however, the JP273v(pEP301) invasion phenotype was not thermally regulated (Table 1). It is possible that expression of *inv* on pEP301 was not regulated by temperature (data not shown) because of a multicopy effect or because this clone may lack all the necessary regulatory sequences upstream of *inv*. Nevertheless, it is clear that *inv* encodes the primary and most efficient factor for invasion of tissue culture cells by *Y. enterocolitica*.

***In Vivo* Invasion by the *inv* Mutant.** Invasion of cultured mammalian cells by *Y. enterocolitica* has been used as a model to reflect penetration of the intestinal mucosa *in vivo*. However, it is not clear whether this model accurately simulates the *in vivo* situation, since bacteria can express a different set of proteins on their surface during proliferation within the intestinal lumen than in culture (16), and the surface of cultured epithelial cells may differ dramatically from that of the intestinal mucosa. Initial invasion *in vivo* was examined by infecting mice intragastrically with either 8081v (wild type) or JP273v (*inv* mutant) grown at 23°C and quantitating the colonization of PP at 6, 18, and 32 hr (Fig. 1). The number of cfu recovered from PP early after infection reflects the efficiency of initial adhesion and invasion by *Y. enterocolitica*. PP from mice infected with 8081v showed a gradual increase in bacterial colonization, with uniformly high bacterial numbers 32 hr postinfection. In contrast, at 6 and 18 hr from $10^{3.4}$ to $10^{7.2}$ fewer JP273v than 8081v cfu were recovered from PP (Fig. 1*A*). Further, mice infected with JP273v at 32 hr fell into two groups, those with high bacterial counts (near wild-type levels) and those with very few or no detectable cfu in PP (Fig. 1*A*). JP273v cells recovered from PP were used to infect a new set of mice to examine whether JP273v passaged through a mouse had regained its ability to invade efficiently *in vivo*, but these JP273v isolates remained defective for invasion (data not shown). When *inv* was reintroduced into JP273v in trans, invasion of PP was restored to

Table 1. Invasion of HEp-2 cells by various *Y. enterocolitica* strains

Strain	*inv* genotype*	% invasion 23°C	% invasion 37°C
8081v	+	29.9 ± 4.76	11.2 ± 1.25
8081v(pEP301)	++	17.1 ± 3.85	14.2 ± 1.81
JP273v	−	0.375 ± 0.010	0.015 ± 0.001
JP273v(pEP1823)	−	0.200 ± 0.010	0.023 ± 0.001
JP273v(pEP301)	++	10.1 ± 2.14	8.14 ± 0.929

Data represent the mean ± SD from three replicates and reflect the results from several experiments.

*+, Single copy of wild-type *inv* gene; ++, multiple copies of wild-type *inv*; −, duplicate, truncated (nonfunctional) copies of *inv*.

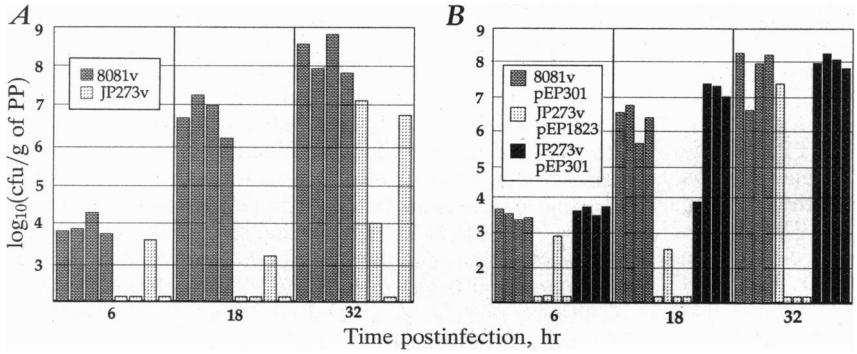

FIG. 1. Colonization of murine PP by the *Y. enterocolitica inv* mutant. BALB/c mice were infected intragastrically with ≈10^8 bacteria, and PP were dissected 6, 18, and 32 hr later. Each bar represents the number of cfu recovered from a single mouse. Plasmid pEP301 is the *inv*-complementing plasmid, and pEP1823 is the vector backbone of pEP301.

wild-type levels (Fig. 1*B*), consistent with the restoration of invasion of cultured epithelial cells (Table 1). JP273v remained defective for invasion of the murine intestine when carrying the vector alone, pEP1823 (Fig. 1*B*). The complementing plasmid pEP301 did not enhance or inhibit the invasive ability of wild-type *Y. enterocolitica* (Fig. 1*B*). These results demonstrate that invasin is necessary for *Y. enterocolitica* to efficiently breach the intestinal epithelium. Further, there was a striking correlation between these results and the tissue culture invasion data.

Virulence of the *inv* Mutant (Oral LD_{50}). The role of invasin in the spread of *Y. enterocolitica* to deeper tissues (liver and spleen) and its role in the subsequent establishment of a systemic infection had not been examined. To determine whether the presence or absence of invasin affects the fate of *Y. enterocolitica* subsequent to mucosal invasion, the oral LD_{50} values for wild-type and *inv* mutant *Y. enterocolitica* were measured. It was expected that the JP273v oral LD_{50} would be increased (i.e., less virulent) relative to wild type, since JP273v was defective for invasion of the murine intestinal epithelium. However, the oral LD_{50}, the average time to death, and the number of cfu recovered from the reticuloendothelial system organs of dead mice were similar for both JP273v and 8081v (Table 2).

Some differences between 8081v and JP273v were observed in their distribution within the reticuloendothelial system. Only 15% of the mice that survived a JP273v infection had cfu in the liver and spleen, compared with 73% for 8081v. In contrast, the liver and spleen from mice fatally infected with JP273v had visible abscesses more often than mice fatally infected with 8081v. The opposite was true for PP, which were easily harvested from mice fatally infected with 8081v, since this organ was usually abscessed. PP from mice fatally infected with JP273v were very difficult to locate, but the few that were found had high bacterial counts because they were abscessed (Table 2). Thus, when JP273v was able to reach the deeper tissues, it was fully capable of causing a fatal infection in the same time frame as 8081v.

Kinetics of Infection. It was not evident why JP273v was only rarely recovered from mice that survived infection yet was as virulent as the wild type as measured by death. It is possible that JP273v was cleared from the deeper tissues within 2 weeks or that JP273v simply did not reach the deeper tissues as often as wild type. If the latter is true, then when the *inv* mutant does eventually traverse the mucosal epithelium, it appears to survive and establish a systemic infection as well as wild type. The LD_{50} measures death as an end point, which does not allow an analysis of the subtle differences between a JP273v and 8081v infection in live mice. Therefore, a kinetic analysis was undertaken to compare the course of infection of wild type and the *inv* mutant.

Mice were infected with 1 LD_{50} (≈5×10^6), and the organs of the reticuloendothelial system were harvested on the days shown in Fig. 2. The wild-type infection showed a clear

Table 2. Oral and i.p. LD_{50} values and *Y. enterocolitica* recovered from organs of fatally infected BALB/c mice

Strain	LD_{50} Oral, no. $\times 10^{-6}$	LD_{50} i.p., no. $\times 10^{-3}$	Recovery, (cfu/g) $\times 10^{-9}$ Liver	Spleen	PP	Mean time to death,* days
8081v	5.64	2.66	0.118	1.36	3.74	7.4
JP273v	3.95	2.43	0.084	0.98	2.66†	7.5

LD_{50} values were determined according to Reed and Muench (17). The cfu values for each organ are averages from 20 mice that died during oral LD_{50} and kinetic assays.
*Range, 5–9 days after infection.
†PP were recovered from only 5 of 20 mice that died from JP273v infection.

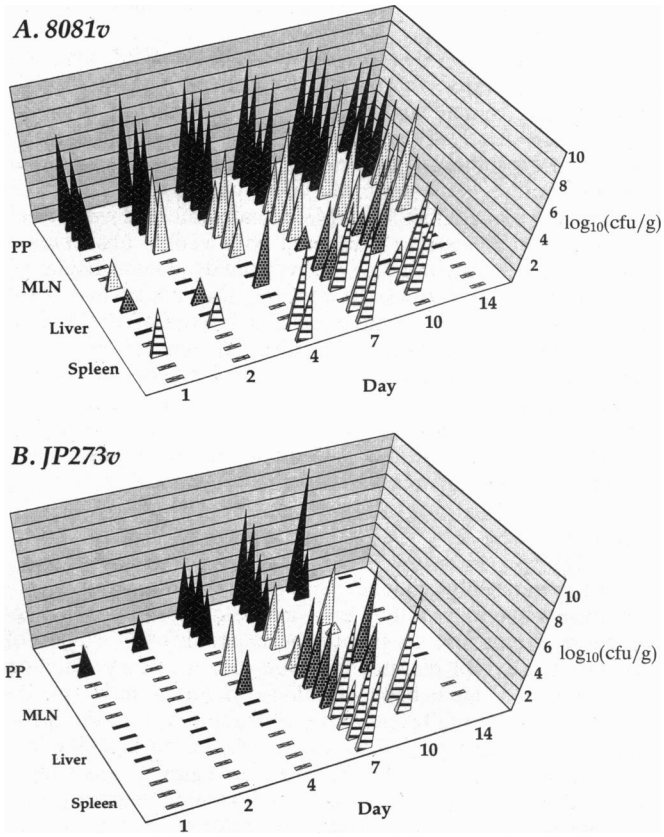

FIG. 2. Kinetics of infection for *Y. enterocolitica* wild type 8081v (*A*) and *inv* mutant JP273v (*B*). BALB/c mice were infected with 1 LD_{50} (≈5×10^6 bacteria). Organs of the reticuloendothelial system were dissected on the days indicated. Each pyramid represents data from a single mouse, and results below the limit of detection are represented as rectangles. Four mice were dissected at each time point, unless fewer than four mice were available. The first pyramid in each group of four, for each organ, represents data from the first mouse; the second pyramid in each group of four, for each organ, represents data from the second mouse, etc.

progression through the reticuloendothelial system; by day 1 it had colonized the PP, by day 4 it had fully colonized the MLN, and by day 7 it had significantly infected the liver and spleen (Fig. 2*A*). From day 4 to 14, 94% of PP, 38% of MLN, 6% of spleens, and 0% of livers were visibly abscessed.

In contrast, JP273v did not consistently infect PP until day 4 and was less efficient at establishing a significant infection of MLN (Fig. 2*B*). However, JP273v colonized the liver and spleen as well as 8081v by day 7 (Fig. 2). The visible abscesses were similar in appearance, but the distribution differed; from day 4 to 14, 8% of PP, 0% of MLN, 33% of spleens, and 33% of livers were abscessed. JP273v was complemented in trans by pEP301, and 8081v was unaffected by pEP301, since these strains exhibited a course of infection similar to wild type (data not shown). Further, the course of infection for JP273v carrying the vector pEP1823 alone was not complemented (data not shown). It is apparent that the lack of functional invasin significantly alters the *Y. enterocolitica* course of infection but not the final outcome (death).

Intraperitoneal LD_{50}. *Y. pseudotuberculosis* invasin binds to several receptors identified as a subset of β_1 integrins, including the fibronectin receptor (4). Phagocytic cells are activated when their fibronectin receptors bind substrate (18), and it has been suggested that the continued presence of invasin on the bacterial cell surface at 37°C may attenuate infection by increasing the susceptibility of bacteria to killing by phagocytic cells (19). If the intestinal barrier is bypassed, then one might predict that the *inv* mutant, by virtue of lacking invasin entirely, could potentially be more virulent than wild type.

The i.p. route of infection bypasses the intestinal invasion step; consequently, the *inv* mutant can be assayed for hypervirulence by measuring the i.p. LD_{50}. However, the i.p. LD_{50} values for 8081v and JP273v were very similar, as was observed for the oral LD_{50} values (Table 2), suggesting that any residual expression of invasin by wild type at 37°C does not alone attenuate infection. Mice fatally infected with either *Y. enterocolitica* strain presented with visible abscesses 5 days postinfection, and the spleens from dead mice displayed similar bacterial counts. In general, the spleen had $\approx 10^9$ recoverable cfu/g of tissue. Similar numbers of JP273v and 8081v cfu were recovered from the spleens of mice that survived i.p. infection, in contrast to survivors of an oral infection. These data are consistent with the observation that JP273v can establish a systemic infection as well as 8081v, but that JP273v has difficulty reaching deeper tissues when it must cross the intestinal epithelium.

DISCUSSION

There is mounting evidence that several pathogenic microorganisms encode multiple invasion pathways that may augment or contribute to pathogenesis at different stages of infection. Several distinct invasion loci that may function independently have been identified in *Salmonella* species (20–24), and two different surface structures from the intracellular parasite *Leishmania* are able to promote uptake into macrophages (25, 26). *Y. enterocolitica* has at least three different invasion factors that function independently (unpublished data; ref. 5). Studies of cultures grown *in vitro* indicate that *inv* is more highly expressed at ambient temperature, presumably to prime the organism for the initiation of infection when it contacts a vulnerable host. *ail*, on the other hand, may be involved later in infection, since it is more highly expressed at 37°C than at lower temperatures and it shows cell-line specificity (M. Wachtel and V.L.M., unpublished data; ref. 5).

In this report we have shown that invasin is a primary invasion factor for *Y. enterocolitica in vitro* and that invasin is necessary for efficient penetration of the intestinal barrier *in vivo* as demonstrated by a marked defect in the ability of *inv* mutants to colonize PP early after infection. This lends support to the relevance of the *in vitro* tissue culture invasion assay as a model for events that occur *in vivo*. However, somewhat unexpectedly a mutation in *inv* had effects on the course of a *Y. enterocolitica* infection beyond simply inhibiting colonization of PP. The wild-type infection showed a gradual progression through the reticuloendothelial system (from PP to MLN to the liver and spleen) that began on day 1 postinfection, but the *inv* mutant showed a delay of 3–4 days before it began to colonize PP. Nevertheless, both the wild type and the *inv* mutant colonized the liver and spleen in the same time frame (by day 7), and the LD_{50} values of wild type and the *inv* mutant were essentially identical, paradoxical findings in light of the data above. In humans, a *Y. enterocolitica* infection is generally a self-limited infection of the gastrointestinal tract with involvement of the PP and the regional lymph nodes. Efficient colonization of the liver and spleen is apparently necessary for a fatal infection in mice; thus, the outcome of infection is more severe in mice than in humans. The gastrointestinal tract disease arising from a *Y. enterocolitica* infection is similar in mice and humans (27); consequently, analysis of PP and MLN early after infection may best reflect the situation in humans. Moreover, LD_{50} determinations, which are dependent on further spread to the liver and spleen (i.e., measure of death), may be unsuitable as a model for the study of gastrointestinal disease. In general, use of the LD_{50} assay as a tool to measure the virulence of pathogens that do not routinely kill their natural host needs to be reevaluated in light of the multifactorial nature of virulence and the availability of more precise models.

The question is how can the *inv* mutant cause death in the same time frame as wild type, yet reach PP at a slower rate and in reduced numbers? In a hypothetical infection model (Fig. 3) that is consistent with our data, a *Y. enterocolitica* infection initiated via the oral route can be operationally divided into two phases: (*i*) adhesion, invasion, and translocation across the intestinal epithelium of the terminal ileum, with multiplication in the PP, and (*ii*) dissemination to deeper tissues with the establishment of a systemic infection. The majority of wild-type *Y. enterocolitica* cells attach to, invade, and colonize PP through the invasin-mediated pathway (Fig. 3, pathway 1). A subpopulation of these bacteria go on to colonize MLN. Subsequently, an even smaller population of bacteria will spread further to cause a systemic infection of the liver and spleen.

The *inv* mutant was able to cross the intestinal epithelium after oral inoculation, albeit less efficiently, as evidenced by its recovery from deeper tissues. The occasional colonization of PP by the *inv* mutant was presumably due to the expression of alternative, less efficient invasion pathways (possibly mediated by Ail, P.D.E. and/or nonspecific uptake by M cells). *Y. enterocolitica* encodes several adhesins that could enable this pathogen to remain in the intestine for extended periods of time. Adhesion would allow the rate of bacterial multiplication to exceed the rate of excretion from the lumen of the gut (28); therefore, sufficient expansion of the *inv* mutant population could provide an opportunity for less efficient invasion pathways to compensate for the lack of invasin-mediated entry. Thus, one or more of the secondary invasion factors, in combination with unidentified host factors, may allow sufficient numbers of the *inv* mutant to invade either through M cells to PP (Fig. 3, pathway 2) or by an unknown pathway through enterocytes (pathway 3). These alternative invasion pathways may also be available to wild type but would make only a minor contribution or would not have an opportunity to come into play, because invasin is such a dominant adhesin and invasion factor.

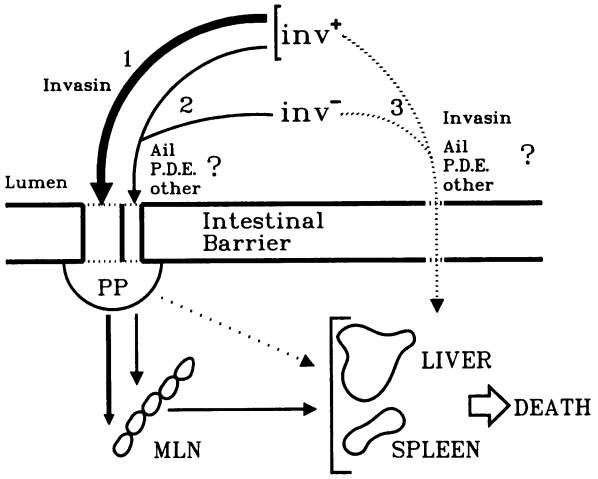

FIG. 3. Hypothetical model of *Y. enterocolitica* invasion pathways and course of infection. *Y. enterocolitica* cells ingested through the oral route need to breach the intestinal barrier. The majority of wild-type organisms expressing invasin (inv^+, thick arrow) will enter through invasin-mediated pathway 1 into PP. Also available to these organisms is the less efficient invasion pathway 2, also into PP and possibly an unknown pathway 3 through enterocytes. Alternative invasion pathways could include one or a combination of the following: Ail-mediated, P.D.E.-mediated, and "other", which could include bacterial factors or host factors (such as M-cell sampling). The *inv* mutant (inv^-) would have only alternative invasion routes 2 and 3 available; thus, initial invasion would be less efficient and severely decreased. Arrow thickness represents the number of bacteria, and the dotted-line arrows indicate unknown, and as yet unproven, pathways. Once the bacteria have crossed the intestinal epithelium, they may spread via the lymphatic system or the bloodstream.

In an oral infection, the *inv* mutant colonizes PP and MLN very poorly yet colonizes the liver and spleen as well as wild type. Thus, invasin appears to have a high affinity for lymphoid tissue, which may inhibit the spread of *Y. enterocolitica* from the initial site of infection. Although fewer *inv* mutant bacteria would be able to invade, those that did breach the intestinal epithelium via an alternative route would be unhindered in their passage through the lymphoid tissue to the liver and spleen (Fig. 3, pathway 2). It is also possible that *Y. enterocolitica* reach the liver and spleen via the bloodstream and that the mutation in *inv* has no effect on this route. Regardless of the route taken by the bacteria, the ability of the *inv* mutant to efficiently colonize the liver and spleen could account for the observation that the wild-type and mutant LD_{50} values are similar. Hence, it is plausible that the absence of invasin will not alter *Y. enterocolitica* virulence significantly as measured by death, but the absence of invasin will alter the pattern of spread and colonization of lymphoid tissue.

The presence of invasin on the *Y. enterocolitica* surface during growth in nature would allow immediate colonization of a susceptible host, presenting this organism with an opportunity to escape general host defenses. A model for the initiation of a naturally occurring infection could be that when contaminated foods containing a low dose of *Y. enterocolitica* are ingested, the presence of invasin determines whether or not an infection is established. Invasin is so efficient at promoting internalization that several orders of magnitude fewer bacteria may suffice to cause gastroenteritis in humans than would be the case in the absence of invasin. For *Y. enterocolitica* lacking invasin, increased bacterial numbers would probably be required to initiate an infection through alternative, less productive invasion pathway(s).

It is apparent that inactivation of any one of the multiple *Y. enterocolitica* virulence determinants will not always lead to a total loss of pathogenicity, yet the course of infection may be drastically altered. From the data reported here it is clear that *inv* encodes an important *Y. enterocolitica* determinant for the initiation of infection and that the absence of invasin causes subtle differences during the systemic phase of infection. Identification of the exact nature of these effects awaits more refined *in vivo* assays and analysis of additional mutations that affect the invasive phenotype of *Y. enterocolitica*.

We thank Susan Valone, Sue Kinder, and Jeff Miller for critically reading the manuscript and for helpful discussions. We are grateful to Bob Leher for helpful discussions about the infection model. We also thank Cyndy Pepe and Marian Wachtel for providing plasmids pCP178 and pMW1823, respectively, and Rafael Martinez for providing the Nal^r variant of *Y. enterocolitica* strain 8081v. This work was supported by U.S. Public Health Service National Research Service Award GM07104 to J.C.P. and by National Institutes of Health Grant AI27342 to V.L.M., who is a Pew Scholar in the Biomedical Sciences.

1. Portnoy, D. A., Moseley, S. L. & Falkow, S. (1981) *Infect. Immun.* **31**, 775–782.
2. Lee, W. H., McGrath, P. P., Carter, P. H. & Eide, E. L. (1977) *Can. J. Microbiol.* **23**, 1714–1722.
3. Isberg, R. R. & Falkow, F. (1985) *Nature (London)* **317**, 262–264.
4. Isberg, R. R. & Leong, J. M. (1990) *Cell* **60**, 861–871.
5. Miller, V. L. & Falkow, S. (1988) *Infect. Immun.* **56**, 1242–1248.
6. Isberg, R. R. (1989) *Infect. Immun.* **57**, 1998–2005.
7. Hanski, C., Naumann, M., Hahn, H. & Riecken, E. O. (1989) *Med. Microbiol. Immunol.* **178**, 289–296.
8. Grützkau, A., Hanski, C., Hahn, H. & Riecken, E. O. (1990) *Gut* **31**, 1011–1015.
9. Rosqvist, R., Skurnik, M. & Wolf-Watz, H. (1988) *Nature (London)* **334**, 522–525.
10. Simon, R., Priefer, U. & Pühler, A. (1983) *Bio/Technology* **1**, 784–791.
11. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 431.
12. Miller, V. L. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170**, 2575–2583.
13. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134**, 1141–1156.
14. Pepe, J. C. & Miller, V. L. (1990) *J. Bacteriol.* **172**, 3780–3789.
15. Miller, V. L., Farmer, J. J., III, Hill, W. E. & Falkow, S. (1989) *Infect. Immun.* **57**, 121–131.
16. Skurnik, M. & Poikonen, K. (1986) *Scand. J. Infect. Dis.* **18**, 355–364.
17. Reed, L. J. & Muench, H. (1938) *Am. J. Hyg.* **27**, 493–497.
18. Wright, S. D., Craigmyle, L. S. & Silverstein, S. C. (1983) *J. Exp. Med.* **158**, 1338–1343.
19. Isberg, R. R. (1990) *Mol. Biol. Med.* **7**, 73–82.
20. Elsinghorst, E. A., Baron, L. S. & Kopecko, D. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5173–5177.
21. Finlay, B. B., Starnbach, M. N., Francis, C. L., Stocker, B. A. D., Chatfield, G., Dougan, G. & Falkow, S. (1988) *Mol. Microbiol.* **2**, 757–763.
22. Galán, J. E. & Curtiss, R., III (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6383–6387.
23. Lee, C., Jones, B. D. & Falkow, S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1847–1851.
24. Stone, B. J., Garcia, C. M., Badger, J. L., Hassett, T., Smith, R. I. F. & Miller, V. L. (1992) *J. Bacteriol.* **174**, 3945–3952.
25. Handman, E. & Goding, J. W. (1985) *EMBO J.* **4**, 329–336.
26. Russell, D. G. & Wright, S. D. (1988) *J. Exp. Med.* **168**, 279–292.
27. Carter, P. B. (1975) *Am. J. Pathol.* **81**, 703–706.
28. Ricciardi, I. D., Pearson, A. D., Suckling, W. G. & Klien, C. (1978) *Infect. Immun.* **21**, 342–344.