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Invasin-Dependent and Invasin-Independent Pathways for Translocation of *Yersinia pseudotuberculosis* across the Peyer's Patch Intestinal Epithelium

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Yersinia pseudotuberculosis **initiates systemic disease after translocation across the intestinal epithelium. Three *Y. pseudotuberculosis* factors, previously identified by their ability to promote association with cultured cells, were evaluated for their relative roles in translocation. To this end, mutants defective for invasin, YadA, or pH 6 antigen were tested for movement from the intestinal lumen into the subepithelium. Within 45 min after introduction of bacteria into the lumen, wild-type bacteria were found in the Peyer's patch. Mutants expressing defective invasin derivatives were unable to promote efficient translocation into the Peyer's patch and instead colonized on the luminal surface of the intestinal epithelium. In particular, a translocation defect was observed in a *Y. pseudotuberculosis* strain that expressed an uptake-defective invasin protein retaining considerable receptor binding activity. To attempt to reduce binding to luminal mucus, *Y. pseudotuberculosis yadA* and *inv yadA* strains were analyzed. Both strains had reduced mucus binding, with the *inv yadA* mutant revealing an alternate uptake pathway that was invasin independent. A mutant defective in the production of the pH 6 antigen adhesin also showed reduced binding to luminal mucus, with specific localization of bacteria in M cells. These results indicate that *Y. pseudotuberculosis* adhesive factors control the site of bacterial interaction within the intestinal environment and that loss of one factor causes drastic changes in the preferred site of localization of the bacterium in this locale.**

Yersinia pseudotuberculosis causes a usually self-limiting gastroenteritis and mesenteric lymphadenitis in humans upon ingestion of contaminated food (4, 37). As is true of all pathogenic *Yersinia* species, this microorganism harbors a 70-kb virulence plasmid that has been shown to be required for pathogenesis (1, 7, 31). After ingestion, the enteropathogenic *Y. pseudotuberculosis* and *Yersinia enterocolitica* pass into the small intestine, where they translocate across the intestinal epithelium at sites of lymphoid tissue in the gut known as the Peyer's patches (13, 37). Both enteropathogens then migrate to the mesenteric lymph nodes and are subsequently found in the liver and spleen, where they replicate extracellularly (23, 32, 34a). For *Y. pseudotuberculosis*, there is some evidence that translocation occurs primarily across M cells in the Peyer's patches (12). This may be central to pathogenesis, as bacteria that do not translocate across the intestinal epithelium will most likely be swept away by intestinal contents and expelled from the host.

Y. pseudotuberculosis and *Y. enterocolitica* are efficiently internalized by cultured cells (2, 3, 18, 25, 38), and several bacterial factors have been implicated as being important for interacting with mammalian cells in culture. Invasin mediates uptake of *Y. pseudotuberculosis* into mammalian cells through binding with very high affinity to a subset of β_1-chain integrins on the mammalian cell surface (17, 18, 19, 27). Five different integrins bind invasin, including the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins (15, 17, 20). Mutational analysis of the C terminus of invasin, which has been shown to be sufficient for binding, has demonstrated that the aspartate residue at position 911 plays an essential role in the binding of integrins by invasin (22). When this amino acid is changed to an alanine, the mutant protein is unable to bind to HEp-2 cells. A more conservative change, to a glutamate, results in an ~10-fold decrease in cell binding compared to that of the wild-type protein (22).

In the absence of invasin, other *Y. pseudotuberculosis* proteins can mediate adhesion or uptake by HEp-2 cells, albeit to a lesser degree. YadA, an adhesin encoded by the *Y. pseudotuberculosis* virulence plasmid, has been shown to bind to collagen, fibronectin, mucus, and mammalian cells (10, 28, 36) and is responsible for the small amount of uptake into cultured cells observed in *Y. pseudotuberculosis inv* strains. There is also some evidence that YadA can bind either directly or indirectly to β_1-chain integrins (2). Another ligand that can bind cells in the absence of invasin is pH 6 antigen, the product of the *psaA* gene, which is expressed by *Y. pseudotuberculosis* at 37°C under acidic conditions (39), although no bacterial uptake occurs after adhesion via this factor (39).

Pepe and Miller (29) showed that *Y. enterocolitica inv* mutants killed mice with the same time course and same 50% lethal dose as wild-type *Y. enterocolitica* after oral inoculation. The *inv* mutant, however, does not colonize the Peyer's patches as efficiently as the wild type, indicating a role for invasin at this step in infection. These data indicate that there is a role for invasin at early times during infection, probably by enhancing the rate of translocation. Once colonization occurs in deeper tissues, this factor seems dispensable in an animal model of infection (33).

The low density of integrin receptors on the apical surface of enterocytes (8, 9) suggests that a minority class of cells with higher receptor density, such as M cells, may be responsible for uptake. M cells, so named because of their irregular, membra-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant feature	Reference or source
E. coli AM2574 (SM10λ*pir thyA*)	*thi thr leu tonA lacY supE recA thyA*::RP4-2-Tc::Mu	34; this study
Y. enterocolitica 8081v	P^+ wild type	V. Miller
Y. pseudotuberculosis		
AM2531	P^+ wild type	13
AM2527	P^- wild type	18
AM2693	P^+ *yadA*::*kan*	M. Skurnik
AM2630 P^+	P^+ *inv*D911A	This study
AM2621 P^+	P^+ *inv*D911E	This study
AM2532	P^+ *inv*::*kan*	18
AM2528	P^- *inv*::*kan*	18
AM2726	P^+ *inv*D911A *yadA*	This study
AM2727	P^+ *inv*D911E *yadA*	This study
AM2770	P^+ *psaA*	This study
pAY01	Amp^r; *sacB ori*TRP4 *ori*R6K	38
pAM203	pAY01 with 1.0-kb *Eco*RV-*Nru*I *inv* fragment; *inv*D911A mutation	This study
pAM204	pAY01 with 1.0-kb *Eco*RV-*Nru*I *inv* fragment; *inv*D911E mutation	This study
pRI285		
pRI285	pBR325 *malE-inv497*	22
pRI203	pBR325 with 4.6-kb *Bam*HI *inv* fragment	18
pAM226	pACYC184 with 4.6-kb *Bam*HI *inv* fragment from pRI203	This study

nous apical surface, are a rare cell type found in the epithelial lining of the Peyer's patches of the intestine (5, 26). They contain a pocket at their basolateral surface in which lymphocytes and macrophages reside, with their presumed function being to sample luminal contents of the intestine and deliver them to their basolateral pockets and thus to the immune system. Microscopic studies previously indicated that *Y. pseudotuberculosis*, like many other bacterial as well as viral pathogens, binds preferentially to the apical surface of M cells on the Peyer's patch surface and not to surrounding brush border epithelial cells (12). The bacterial factor mediating this binding has not been identified. The purpose of the present work is to determine the role of known *Y. pseudotuberculosis* factors in translocation via M cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in these studies are described in Table 1. *Y. pseudotuberculosis* and *Escherichia coli* strains were grown in L broth or on Luria-Bertani agar containing the appropriate antibiotics at the following concentrations (in micrograms per milliliter): ampicillin, 50 for *Y. pseudotuberculosis* and 100 for *E. coli*; and chloramphenicol, 20 for both. Bacteria were prepared for infection studies as follows. For in vitro experiments, *Y. pseudotuberculosis* strains were grown overnight at 28°C to saturation; the bacteria were then washed and diluted 1:100 in phosphate-buffered saline (PBS), and 10 μl of diluted bacteria was added to HEp-2 cells in wells of 24-well plates. For in vivo Peyer's patch invasion experiments, *Y. pseudotuberculosis* strains were grown overnight at 28°C to saturation, washed once with PBS, and diluted to an A_{600} of ≈1 in PBS. A 100- to 150-μl sample of this suspension was used to feed mice (see below). For ligated-loop experiments, *Y. pseudotuberculosis* strains were grown overnight at 28°C and then subcultured 1:50 and grown for 3 to 4 h longer. The bacteria were then washed once with PBS, resuspended in 1/10 volume of PBS containing 0.3 mg of 5- and 6-carboxytetramethylrhodamine succinimidyl ester (TAMRA) (Molecular Probes, Eugene, Oreg.) per ml, and incubated with shaking at 28°C for 1 to 2 h longer. The suspensions were then washed six times with PBS and examined by fluorescence microscopy. A 50-μl volume of this suspension (~5×10^8 bacteria) was used to infect mouse ligated loops, as described below.

Construction of invasin point mutants. *Y. pseudotuberculosis* strains with point mutations at *inv*D911 were constructed as follows. Suicide plasmids pAM203 and pAM204 are pAY01 derivatives (38) carrying the *E. coli sacB* gene and the 1.0-kb *Eco*RV-*Nru*I fragment of invasin from plasmid pRI285 (22) with the *inv*D911A and *inv*D911E mutations, respectively. These plasmids were introduced into wild-type *Y. pseudotuberculosis* by conjugation with an *E. coli* donor strain, AM2574 (SM10λ*pir thyA*). After selection of transconjugants on minimal agar plates containing ampicillin, clones were selected for loss of the plasmid by being plated on L-agar plates containing sucrose and were further screened for ampicillin sensitivity. Candidate mutant colonies were screened for binding to HEp-2 cells, as described elsewhere (21), and for promoting uptake by HEp-2 cells (16). Clones which had lost the uptake ability were screened for loss of plasmid sequences by Southern blot analysis, and the region around each of the mutations was sequenced after PCR amplification using *inv*-specific primers at nucleotide 2798 (5′-CCGCAGGTTGATACCCTGATACTGAGT-3′) and nucleotide 3322 (5′-TTCACCAGATTGCCAATCAGA-3′) (18).

Invasin purification. Overexpressed MBP-Inv497 fusion protein from plasmid pRI285 was purified by DEAE chromatography, as previously described (22). In the present studies, the MBP-Inv497 fusion was cleaved with Factor Xa (New England Biolabs, Beverly, Mass.), and the Inv497 moiety was isolated from MBP by passage of the cleavage reaction mixture over a cross-linked amylose column as described previously (22).

Cell culture and in vitro infection experiments. HEp-2 cells used for cell binding and uptake assays were maintained in RPMI 1640 medium containing 5% newborn calf serum and 20 mM L-glutamine at 37°C with 5% CO_2. In preparation for either assay, HEp-2 cells were treated with trypsin (Gibco-BRL, Grand Island, N.Y.) for 10 min at room temperature and then replated in wells of 24-well dishes and grown to 70 to 80% confluency. Bacterial uptake was assessed as the percentage of bacteria which survived killing by the addition of the antibiotic gentamicin to the external medium, as described elsewhere (16). The efficiency of bacterial binding to HEp-2 cells was quantitated by counting bacteria bound per HEp-2 cell in Giemsa-stained preparations, as described previously (16). Experiments were routinely done in triplicate or quadruplicate.

Mouse ligated-loop infections. Six- to eight-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were starved for 4 h prior to infections and then were anesthetized with 2,2,2-tribromoethanol (Avertin; 250 mg/kg; Aldrich, Milwaukee, Wis.) administered intraperitoneally. After externalization of a short segment of small intestine, ~1-cm-long ligated loops were tied around a Peyer's patch, and ~5×10^8 CFU of TAMRA-labeled *Y. pseudotuberculosis* was injected into each loop by using a 27-gauge needle attached to a 1-ml syringe. The loop was returned to the abdominal cavity, and the infection was allowed to proceed for 20 to 60 min, at which time the mice were euthanized by CO_2 asphyxiation.

For immunofluorescence studies, infected Peyer's patch tissue was excised, rinsed gently with PBS, and then immediately placed in cold 2% paraformaldehyde in PBS for 2 h. After fixation, the tissue was equilibrated in 25% sucrose–0.1% formaldehyde overnight at 0°C, embedded in TBS tissue freezing medium (Triangle Biomedical Sciences, Durham, N.C.), and 2- to 6-μm-thick frozen sections were cut on a cryostat (International Equipment Co.). Sections were collected on coated glass slides, and TBS medium was dissolved by incubating the sections in distilled water. In some experiments, sections were probed with various reagents as described below, or sections were mounted on coverslips and examined by fluorescence microscopy on a Zeiss Axioskop or a Zeiss confocal microscope. Each experiment was performed at least three times.

TABLE 2. In vitro phenotypes of *Y. pseudotuberculosis* strains

Strain	Genotype[a]	HEp-2 cell binding[b]	% Uptake[c]
AM2527	P^- wild type	32.9 ± 1.3	23.7 ± 5.6
AM2621	P^- *inv*D911E	13.05 ± 0.73	0.045 ± 0.009
AM2630	P^- *inv*D911A	4.86 ± 0.37	0.058 ± 0.008
AM2528	P^- *inv*::*kan*	0.92 ± 0.09	0.22 ± 0.029

[a] All strains have been cured of the virulence plasmid (P^-).

[b] Number of bacteria bound per HEp-2 cell as described in reference 16. The assay was performed on triplicate coverslips, and 150 cells were counted per coverslip. Data are means ± standard errors of the means.

[c] Percentage of input bacteria surviving gentamicin killing, as described in reference 16. The assay was performed with triplicate wells. Data are means ± standard errors of the means.

Peyer's patch invasion experiments. Bacteria used for intragastric feeding experiments were prepared as described above. Six- to eight-week-old female BALB/c mice were fed 100 to 150 μl of a prepared bacterial suspension by using either 0.76-mm-diameter Intramedic tubing (Becton-Dickinson Co., Sparks, Md.) attached to a 21-gauge needle or a 22-gauge stainless steel ball-tipped feeding needle (Harvard Apparatus, Inc., Natick, Mass.) attached to a 1-ml syringe. Mice were given food and water ad libitum prior to and following feeding. At appropriate times after infection, mice were euthanized by CO_2 asphyxiation, and Peyer's patches of the small intestine were excised, rinsed with sterile PBS, and incubated in PBS containing 50 μg of gentamicin per ml in order to kill any bacteria which had adhered to the luminal surface of the Peyer's patch or to the villi. After 30 min of incubation with gentamicin, the tissues were washed three times with PBS and homogenized in 100 μl of PBS. Bacteria recovered from these tissues were quantitated by being plated on MacConkey-lactose agar plates at 28°C. As a control, non-Peyer's patch segments of small intestine were also removed from infected animals and treated with gentamicin as described above. Each experiment was performed at least three times.

Electron microscopy. Infected-mouse Peyer's patch samples were prepared for electron microscopy as follows. Excised tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 2 h at 4°C and then washed three times (15 min each) in the same buffer. After a postfixation step with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h on ice, the samples were washed three times for 15 min each with cacodylate buffer and dehydrated with increasing concentrations of ethanol (30 to 100%) for 10 min each. The samples were then subjected to infiltration with propylene oxide for 15 min at room temperature followed by successive incubations in propylene oxide-Epon (13 g of DDSA, 21 g of polybed 812 resin, 9 g of NMA, 0.725 ml of DMP-30) mixtures (1:1 and 1:2) and in 100% Epon. Finally, the tissues were placed in fresh Epon in molds and allowed to harden at 60°C for 24 to 48 h. Thin sections were cut on a Reichert-Jung Ultracut microtome with glass knives and viewed by transmission electron microscopy on a Philips CM-10 electron microscope.

Immunofluorescence microscopy. Mice were sacrificed by CO_2 asphyxiation, and Peyer's patches were excised, rinsed with PBS, and fixed in 2% paraformaldehyde in PBS for 2 h at 0°C. The tissues were then infiltrated with 25% sucrose–0.1% formaldehyde and stored at −80°C until they could be processed. Immediately after thawing on ice, the tissues were embedded in TBS medium and frozen, and 2- to 6-μm-thick sections were cut on a cryostat with a stainless steel knife. Sections collected on glass slides were treated first with distilled water to dissolve TBS medium and then with 50 mM NH_4Cl for 10 min in a humid box at room temperature to block reactive aldehydes. Nonspecific protein binding sites were blocked by incubation of sections with 0.2% gelatin in PBS for 30 min at room temperature. For some experiments, sections were then probed with lectin UEA-1, which recognizes the fucose α(1-2)Gal linkage in mucus and on M cells in the Peyer's patch surface (6, 11, 14), conjugated to fluorescein isothiocyanate (FITC) or TRITC at 5 μg/ml (Sigma). These primary reagents were incubated with the sections for 2 h at room temperature in a humid box. The slides were then washed three times for 5 min each with PBS and then once with distilled water, and coverslips were mounted and sealed. Sections were examined by fluorescence by confocal microscopy or with a Zeiss Axioskop. Images of sections collected by confocal microscopy were processed on a Macintosh 9500 computer using IPLab Spectrum software (Signal Analytics).

RESULTS

In vitro phenotypes of *inv*D911A and *inv*D911E mutants. As invasin is able to promote both cellular adhesion and uptake, mutants which had differential effects on these two activities were analyzed to determine which property played a role in intestinal colonization. Previous work (22) demonstrated that residue D911 was critical for the binding of purified invasin to integrin receptors. An aspartate-to-alanine change at this residue results in a protein with no binding activity, whereas an aspartate-to-glutamate change results in a protein with residual binding activity, based on the ability of pure protein to bind mammalian cells. These two mutations were separately crossed onto the *Y. pseudotuberculosis* chromosome and confirmed by Southern analysis and DNA sequencing (Materials and Methods). The resulting mutants were analyzed for their efficiency of binding and internalization by HEp-2 cells in culture (Table 2) to determine if these activities when expressed in the parental *Y. pseudotuberculosis* strain were similar to results obtained by using purified protein preparations (22).

Wild-type *Y. pseudotuberculosis* cured of its virulence plasmid [*Y. pseudotuberculosis* III (P^-)] showed efficient attachment to and uptake by HEp-2 cells, in an invasin-dependent fashion. An *inv*::*kan* (P^-) insertion mutant averaged less than one bacterium bound per HEp-2 cell and a 100-fold decrease in uptake compared to that of the wild type. Whereas *inv* point mutants were as defective for uptake as the *inv* null mutant, they promoted cell binding at levels that were intermediate between those of the wild-type strain and the *inv*::*kan* strain (Table 2). *Y. pseudotuberculosis* strains harboring either of the mutant *inv* alleles produced full-length invasin derivatives that were exported to the bacterial surface at wild-type levels, on the basis of immunoblotting and immunofluorescence results (data not shown). Therefore, the differences in the levels of binding for the mutant strains allowed us to determine whether translocation in vivo was dependent on high-affinity binding and internalization within host cells (wild-type strain) or whether binding in the absence of uptake was sufficient to promote translocation (*inv*D911E mutant).

In vivo phenotypes of *inv*D911A and *inv*D911E mutants. Strains harboring the *inv*D911A and *inv*D911E mutations were used to infect mice orally to determine if residual cell binding by invasin was sufficient to allow translocation across the intestinal epithelium. To ensure that any defects observed were due to translocation and not survival within the Peyer's patch, short-term viable-count experiments were performed (after <2

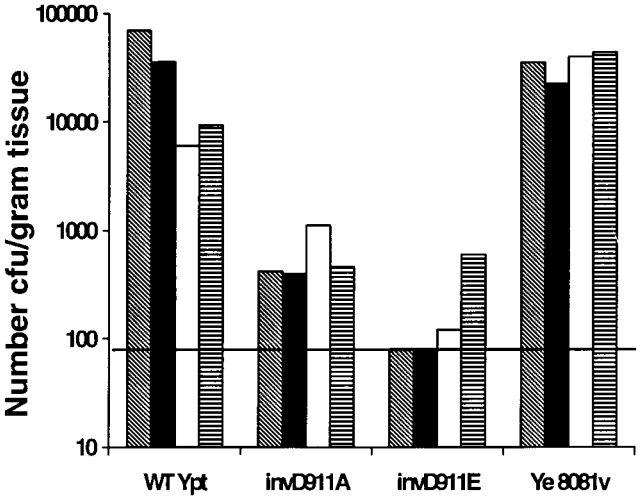


FIG. 1. Peyer's patch invasion by *inv* mutants of *Y. pseudotuberculosis*. Four BALB/c mice per strain tested were infected intragastrically with 5×10^8 bacteria and sacrificed after 90 min. Peyer's patches were harvested, and the number of bacteria per gram of Peyer's patch tissue was determined for each mouse (Materials and Methods). The limit of detection for this assay (80 CFU/g of tissue) is indicated (horizontal line). This experiment was performed at least three times in triplicate or quadruplicate; the results shown represent a typical experiment. WT Ypt, wild-type *Y. pseudotuberculosis*; Ye, *Y. enterocolitica*.

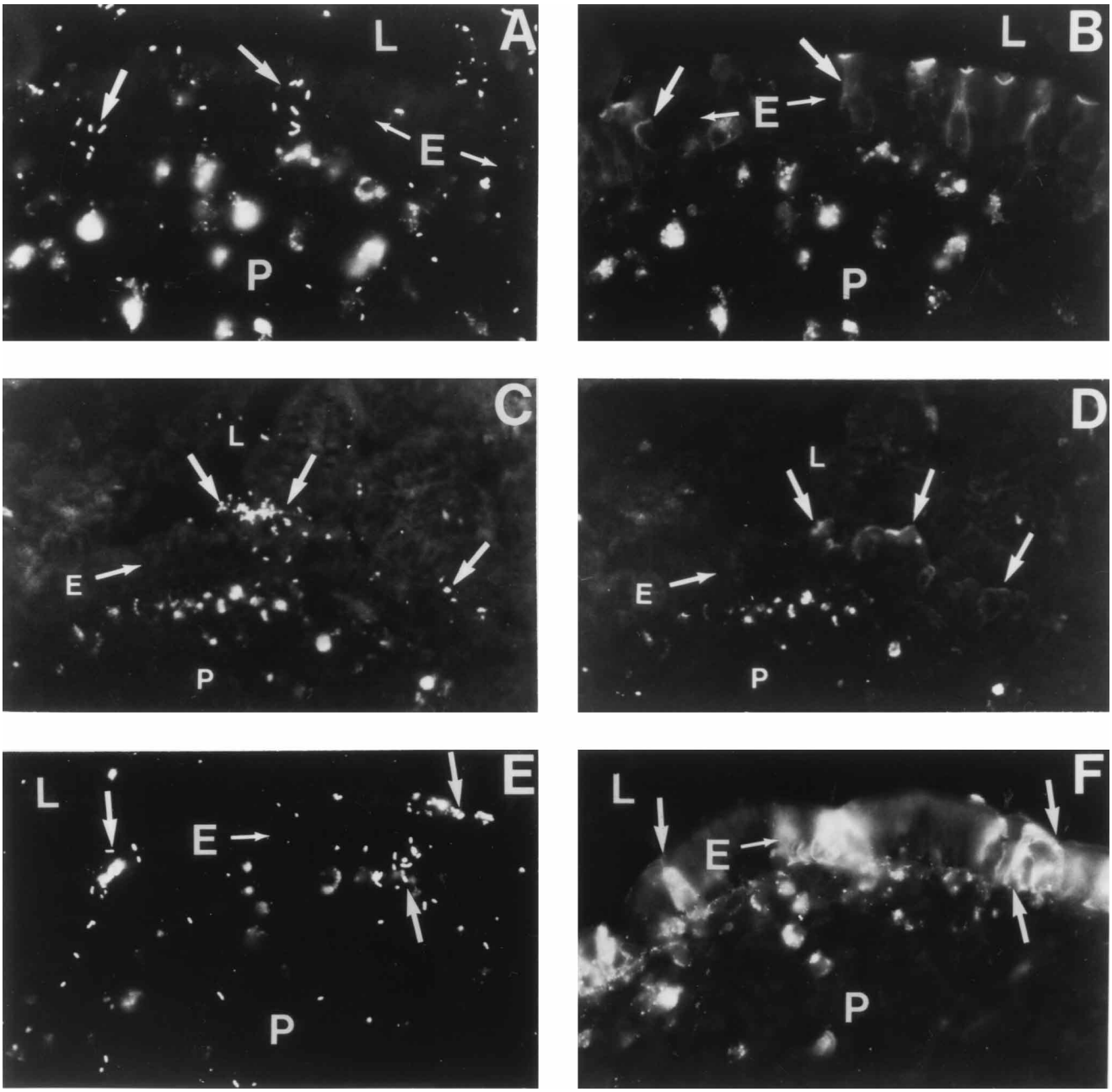
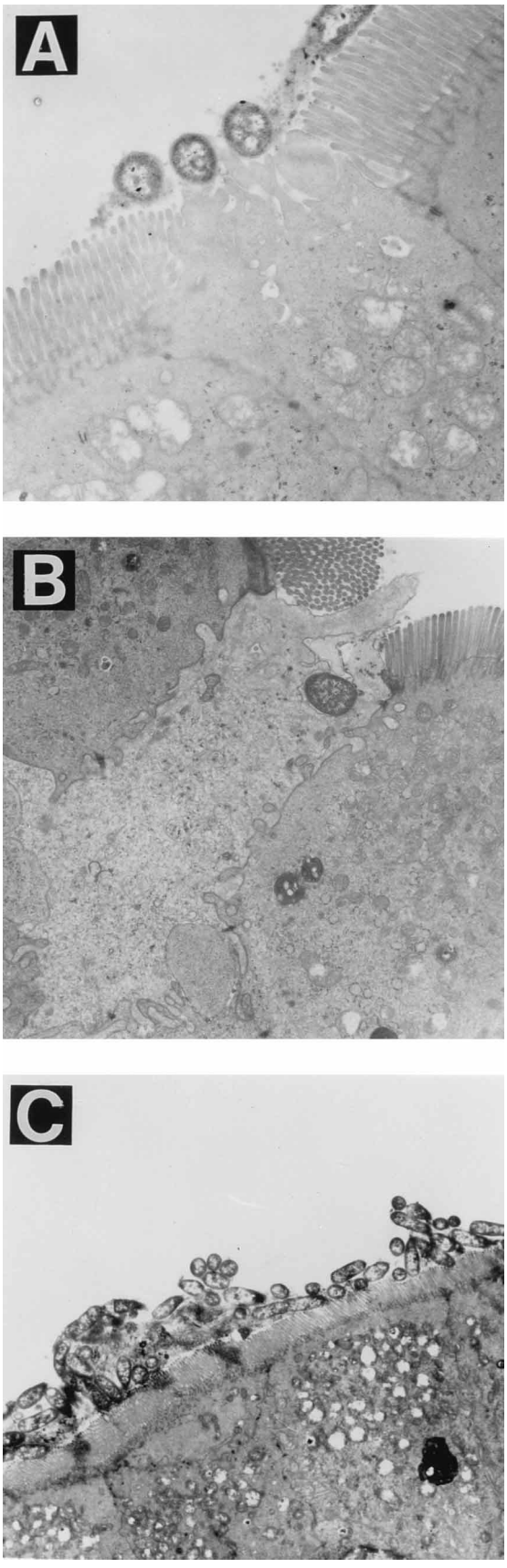


FIG. 2. Infection of mouse ligated loops with *Y. pseudotuberculosis* mutants. Mouse ligated loops were prepared as described in Materials and Methods and infected with 10^8 TAMRA-labeled bacteria. After 45 min, Peyer's patch tissue was excised and prepared for microscopy. Fluorescence signals seen in both rhodamine and fluorescein channels are autofluorescent lymphocytes in the subepithelial regions. (A) Infection with wild-type *Y. pseudotuberculosis* AM2531. (B) Same field as in panel A, after tissue sections were probed with the lectin UEA-1 conjugated to FITC. M-cell staining with UEA-1 is limited to the luminal side of the cells. (C) Infection with *Y. pseudotuberculosis* AM2630 (*inv*D911A). (D) Same field as in panel C, after the tissue was probed with the lectin UEA-1 conjugated to FITC. (E) Infection with AM2726 (*inv*D911A *yadA*). (F) Same field as in panel E after the tissue was probed with lectin UEA-1 conjugated to FITC. Panels A, C, and E were examined under the rhodamine channel; panels B, D, and F were examined under the fluorescein channel. The following regions on the tissues are indicated (arrows): L, intestinal lumen; E, epithelial cell layer; P, Peyer's patch. Bacteria in both the rhodamine-stained and the UEA-1-stained samples are also indicated (arrows).

h of infection), and additionally, infected tissues were examined by microscopy, which does not require bacterial viability for detection of translocated organisms.

Significant numbers of wild-type bacteria were within Peyer's patch tissue 2 h after mice were fed 10^8 bacteria, as determined by a viable-count assay (Fig. 1). This was similar to results with the wild-type *Y. enterocolitica* strain 8081v, used in a previous study (29) (Fig. 1). In contrast, the two *inv* point mutants gave yields that were 100- to 1,000-fold lower than that of the wild type (Fig. 1). In a typical experiment, no bacteria were recovered from two of the four mice infected with the *inv*D911E mutant, consistent with the results reported for a *Y. enterocolitica inv* null mutant (29, 30). These results indicate that the *inv* point mutants were defective for Peyer's patch colonization. Furthermore, an *inv* mutant that retains considerable adhesive activity (D911E) but is unable to promote



uptake in cell culture is as defective for this property as a mutant lacking both activities (D911A; Fig. 1).

To demonstrate that the bacteria recovered from infection of the mouse Peyer's patches were not *inv*$^+$ revertants, four colonies from each strain were examined, and all were defective for HEp-2 cell internalization (data not shown). In addition, the decrease in Peyer's patch colonization observed for the *inv* point mutants could be complemented by the addition of an *inv*-containing plasmid, pAM226, to these strains (data not shown), indicating that the mutations in invasin are responsible for this defect.

Analysis of *Y. pseudotuberculosis* infection of mouse ligated loops. To investigate whether defective colonization of Peyer's patches by the *inv* mutant strains was due to a defect in translocation or survival, mouse ligated loops were inoculated with rhodamine-labeled bacteria and examined by fluorescence microscopy of fixed sections prepared 45 min after infection. As shown in Fig. 2A, the wild-type *Y. pseudotuberculosis* strain was able to translocate across the Peyer's patch surface, with bacteria seen deep within this tissue (Fig. 2A). In contrast, the *inv*D911A and *inv*D911E mutants were seen caught within villi, not bound to the Peyer's patch surface and not at sites within the lymphoid tissue of the Peyer's patch (*inv*D911A; Fig. 2C). From these results, we conclude that the *inv* point mutants do not translocate across the Peyer's patch surface.

Examination by electron microscopy of infected Peyer's patch sections was performed to identify the cause of the *inv* mutants' block in translocation (Fig. 3). Most of the bacteria observed in the wild-type *Y. pseudotuberculosis* infection penetrated the Peyer's patch tissue by 45 min after inoculation, although occasionally bacteria can be seen specifically adhering to or entering the apical surface of cells which morphologically resemble M cells (Fig. 3A and B). When infected tissues were viewed by electron microscopy, *Y. pseudotuberculosis* *inv*D911A bacteria were seen caught in a web of what appears to be intestinal mucus, adhering to the Peyer's patch surface but not bound to or invading M cells or brush border cells (Fig. 3C). The same phenomenon was observed for the *inv*D911E mutant (not shown).

To determine whether the matrix associated with the *inv* mutants in the lumen was intestinal mucus, sections of Peyer's patch tissue infected with *Y. pseudotuberculosis* *inv*D911A were probed with the lectin UEA-1, which had previously been shown to bind M cells as well as mucus or glycocalyx by recognition of an L-fucose linkage (6, 11, 14). UEA-1 recognized M cells overlying the Peyer's patch, as well as mucus in the spaces between the villi and the Peyer's patch surface (Fig. 2B and D). Translocating wild-type bacteria colocalized with M cells in the epithelial cell layer, shown as luminal staining with UEA-1 (Fig. 2B). It is clear that the *inv*D911A mutant colocalized with mucus or glycocalyx (Fig. 2D, luminal staining of M cells with UEA-1), consistent with the electron microscopy data. These results suggest that the *inv* point mutants were unable to translocate across M cells at the Peyer's patch surface, perhaps due to their being trapped by intestinal mucus.

Phenotypes of *inv yadA* double mutants in vitro and in vivo. The presence of large numbers of bacteria trapped in mucus (or glycocalyx) during an infection by the *inv* point mutants indicated that in the absence of functional invasin, another *Y.*

FIG. 3. Electron microscopy of infected Peyer's patch tissue. Mouse ligated loops were prepared as described in the text and infected with 10^8 bacteria. After 45 to 60 min, the tissue was excised and processed for electron microscopy. (A and B) Infection with wild-type AM2531; (C) infection with AM2630 (*inv*D911A).

TABLE 3. Uptake of *Y. pseudotuberculosis inv yadA* mutants by HEp-2 cells

Strain	Genotype[a]	% Uptake[b]
AM2531	P^+ *inv*$^+$ *yadA*$^+$	1.34 ± 0.13
AM2693	P^+ *inv*$^+$ *yadA*	1.24 ± 0.18
AM2630	P^+ *inv*D911A *yadA*$^+$	0.38 ± 0.03
AM2726	P^+ *inv*D911A *yadA*	0.029 ± 0.004
AM2621	P^+ *inv*D911E *yadA*$^+$	0.24 ± 0.038
AM2727	P^+ *inv*D911E *yadA*	0.014 ± 0.003

[a] All strains contain either the virulence plasmid (P^+) or the *yadA*::Kan derivative of the virulence plasmid p037. The percent uptake observed was lowered by the presence of the 70-kb virulence plasmid in these strains, as expected from the presence of antiphagocytic factors encoded by this plasmid (7, 35).

[b] Percentage of input bacteria surviving gentamicin killing, as described in reference 30. The assay was performed with triplicate wells. Data are means ± standard errors of the means.

pseudotuberculosis adhesin was the dominant ligand. A likely candidate for such an adhesin is YadA, a surface protein of *Y. pseudotuberculosis* that had previously been shown to bind collagen as well as mucus (10, 28).

Y. pseudotuberculosis yadA derivatives harboring the *inv*D911A or *inv*D911E mutation were constructed and characterized for the effect of this lesion on uptake by HEp-2 cells. Inv$^+$ *Y. pseudotuberculosis* showed similar levels of uptake by cultured cells in the presence or absence of YadA (Table 3; 1.34 and 1.24%, respectively). The *inv* point mutants showed significantly less uptake (0.38% for *inv*D911A and 0.24% for *inv*D911E), whereas the *inv yadA* double mutants were 10-fold less efficient at uptake by HEp-2 cells than either single mutant alone (Table 3).

The behavior of the *inv yadA* double mutant in a Peyer's patch invasion assay was examined. Strikingly, the double mutant was more invasive for the Peyer's patch than the single *inv* mutant (Fig. 4). Mice infected with the *inv yadA* double mutant AM2726 yielded approximately 5×10^3 to 1×10^4 bacteria per g of Peyer's patch tissue. The double mutant was able to efficiently access the Peyer's patch surface and translocate (Fig. 2E and F) and was readily visible by fluorescence microscopy within the Peyer's patch. In contrast, for the strain harboring the single *inv*D911A mutation, only ~500 bacteria were recovered per g of tissue from each of two mice, whereas no bacteria were recovered from two other mice infected with this strain (Fig. 4A), consistent with previous results (Fig. 1). Even the *yadA* mutant AM2693 reproducibly colonized the Peyer's patches somewhat more efficiently than the wild-type strain. In the experiment whose results are displayed, Peyer's patches from all four mice infected with the *yadA* mutant yielded 3- to 10-fold more bacteria per g of tissue than did the wild-type strain (Fig. 4B). Microscopically, the *inv*$^+$ *yadA* mutant was seen to associate with the Peyer's patch surface but not to colocalize with intestinal mucus. Large numbers of *inv*$^+$ *yadA* mutant bacteria had translocated by 45 min postinfection (Fig. 5A versus B). The mice with wild-type infection, in addition to having translocated bacteria, had significantly more bacteria that were luminally adherent (Fig. 5A).

We conclude from these experiments that the *inv yadA* double mutant is more invasive in vivo than bacteria harboring only the *inv* mutation. Presumably, the *inv* mutants were competent for translocation when mucus binding by YadA was eliminated.

In vivo phenotypes of *psaA* mutants of *Y. pseudotuberculosis*. Since surprising changes in bacterial localization were generated by mutations in adhesive factors, we examined the role of another in vitro-identified adhesin in Peyer's patch colonization. It has recently been shown that the *psaA* gene of *Y. pseudotuberculosis*, encoding pH 6 antigen, mediates thermoinducible binding to cells in the absence of the virulence plasmid (39). A *psaA* mutant containing the virulence plasmid was constructed and tested for penetration of Peyer's patches. As shown in Fig. 6, the *psaA* mutant showed the same ability as wild-type *Y. pseudotuberculosis* to colonize Peyer's patches at 2 h postinfection, as determined by a viable-count assay. When this strain was examined in a ligated-loop infection, however, the *psaA* mutant was found to have a very striking phenotype: this mutant appeared to bind and be taken up exclusively by M cells at the Peyer's patch surface, as demonstrated by colocalization of TAMRA-labeled bacteria within UEA-1–FITC-la-

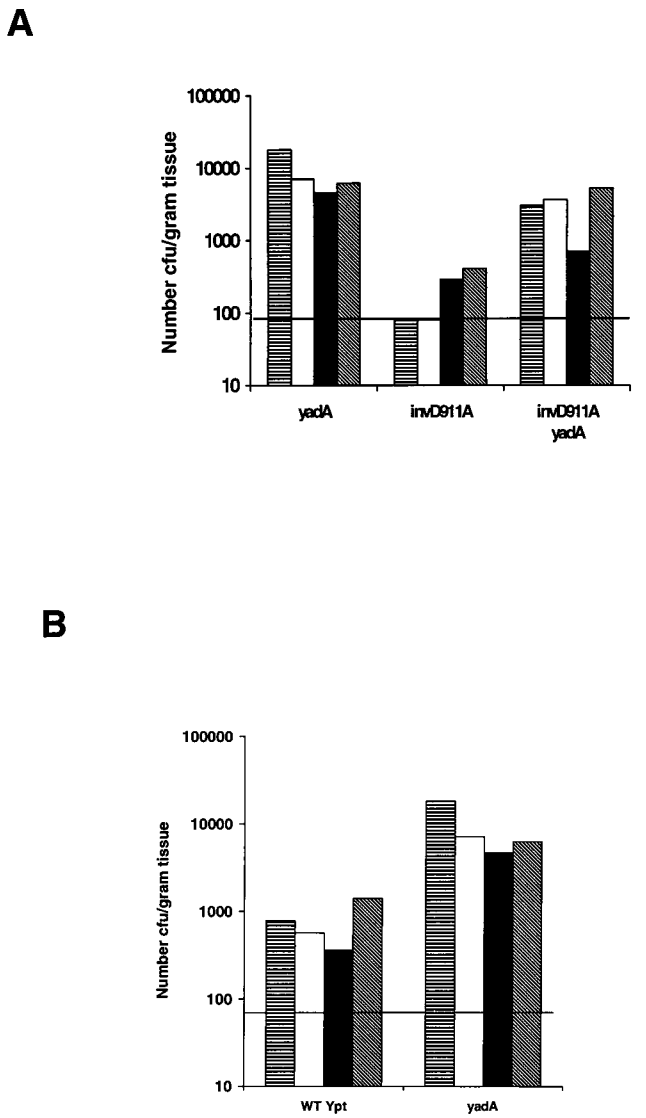


FIG. 4. Peyer's patch invasion by *Y. pseudotuberculosis inv yadA*. Four BALB/c mice per strain tested were infected intragastrically with 5×10^8 bacteria and euthanized after 90 min. Peyer's patches were harvested, and the number of bacteria per gram of Peyer's patch tissue was determined for each mouse. The horizontal line represents the limit of detection for this assay (80 CFU/g of tissue). These experiments were repeated at least three times, and the results shown are representative of typical experiments. These experiments were performed on different days from those whose results are shown in Fig. 1. WT Ypt, wild-type *Y. pseudotuberculosis*.

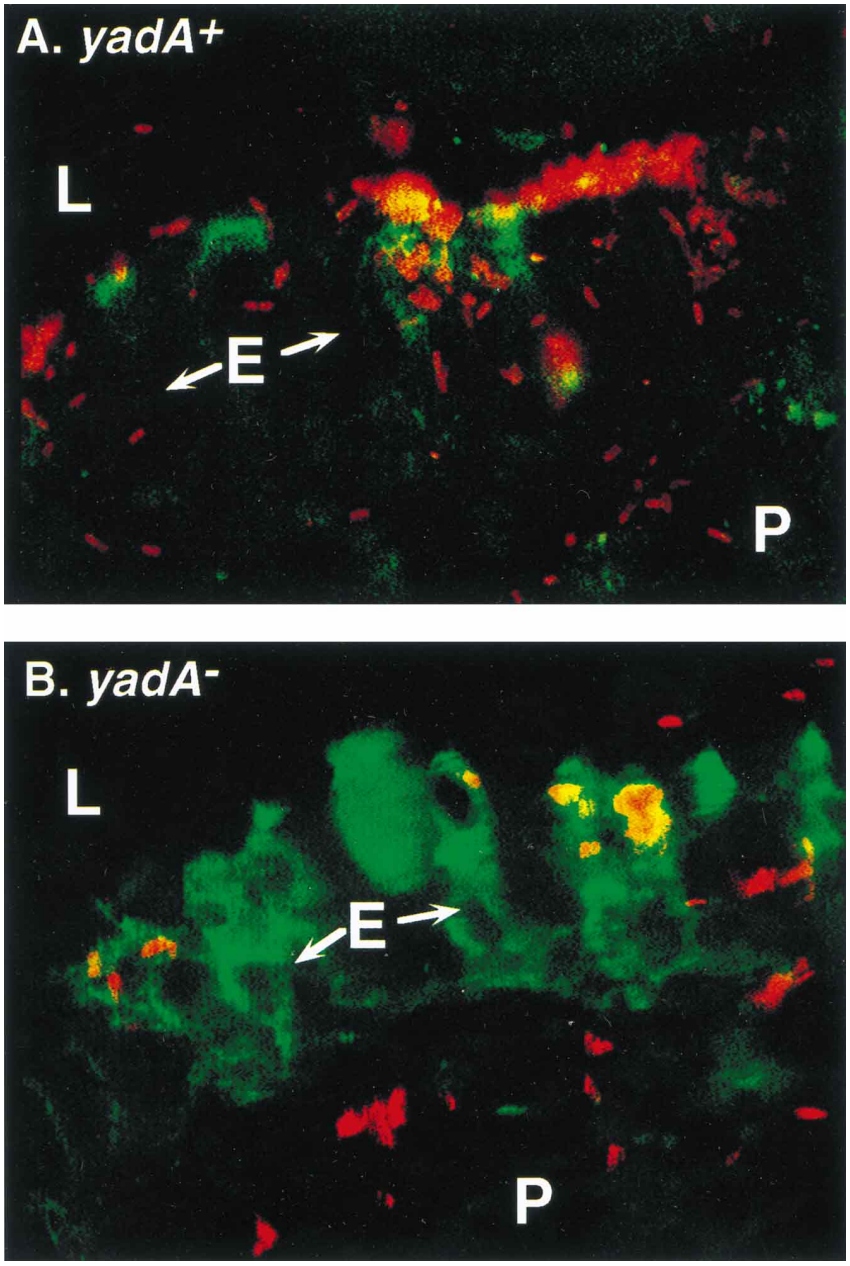


FIG. 5. Confocal fluorescence micrographs of infected-mouse Peyer's patch tissue. Mouse ligated loops were prepared and infected as described in Materials and Methods, and after 45 min the tissue was excised and processed for fluorescence microscopy. The bacteria had been labeled prior to infection with rhodamine succinimidyl ester, and M cells and mucus have been stained with lectin UEA-1 conjugated to FITC. Sites of colocalization of the two markers appear yellow. (A) Infection with wild-type AM2531; (B) infection with *yadA* mutant AM2693. E, epithelial cell layer; L, intestinal lumen; P, Peyer's patch; C, clumps of bacteria in intestinal lumen.

beled M cells (Fig. 7), and showed very little diffuse adherence to the luminal surface of the intestinal epithelium.

DISCUSSION

We have characterized translocation of *Y. pseudotuberculosis* across the intestinal epithelium shortly after oral inoculation. In an otherwise wild-type organism, invasin was shown to be required for efficient localization of viable bacteria within the Peyer's patch, consistent with data from Pepe and Miller for the related *Y. enterocolitica* species (29). Invasin could be required for translocation into, or survival within, the Peyer's patch. To distinguish between these possibilities, short infection times were used, as was fluorescence microscopy, which does not require survival of the organism for bacteria to be observed localized in the Peyer's patch. *Y. pseudotuberculosis inv* mutants were defective for localization in this site, on the basis of visualization of microorganisms 45 min after their introduction into ligated intestinal loops or of viable-count assays performed 90 min after oral inoculation. These data argue that a defect in survival is not sufficient to explain the reduced colonization of Peyer's patches by *inv* mutants.

It is conceivable that invasin allows *Y. pseudotuberculosis* to adhere to the luminal surface of the Peyer's patch without

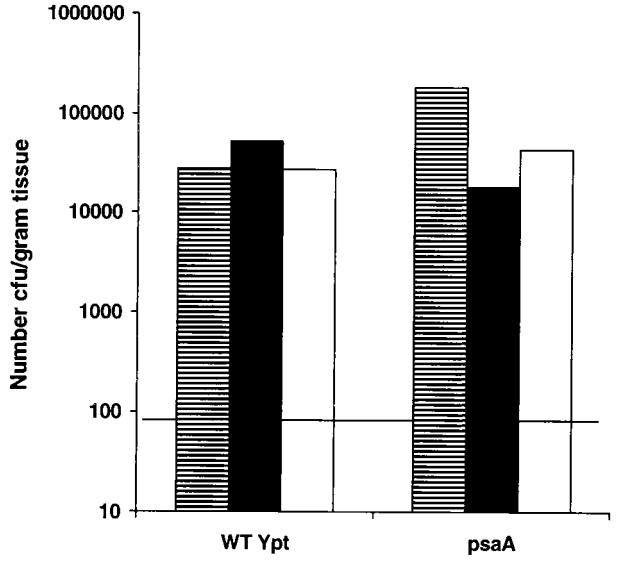


FIG. 6. Peyer's patch invasion by *psaA* mutants of *Y. pseudotuberculosis*. Three BALB/c mice per strain tested were infected intragastrically with 5×10^8 bacteria, and the mice were euthanized at 2 h postinfection. Peyer's patches were harvested, and the number of bacteria per gram of tissue was determined for each mouse. The limit of detection for this assay is indicated (horizontal line). This experiment was performed at least three times, and the results shown are representative of a typical experiment. WT Ypt, wild-type *Y. pseudotuberculosis*.

directly promoting internalization of bacteria by cells. To attempt to address this issue, a mutant derivative of invasin (D911E) that retained considerable receptor binding activity, but which failed to promote uptake of bacteria into cultured cells, was used. This mutant was highly defective for translocation into the Peyer's patch, although it could promote relatively efficient invasin-dependent adhesion to mammalian cells (Table 2). The movement of wild-type *Y. pseudotuberculosis* across the epithelium appears to be a direct result of the ability of invasin to promote bacterial entry into cells.

Previous results on the uptake of *Y. pseudotuberculosis* into cultured mammalian cells showed that the YadA protein could promote low-efficiency internalization in the absence of invasin expression (38). Our translocation assays in the mouse indicated a very different role for YadA during intestinal colonization. Lack of invasin expression in an otherwise wild-type microorganism led to striking colonization of the epithelial cell surface in patches that appeared to be rich in mucus. This colonization pattern was dependent on the presence of YadA protein, as *Y. pseudotuberculosis yadA* or *yadA inv* derivatives were rarely seen on the apical epithelial surface (Fig. 2 and 5). These results, combined with the low efficiency of translocation of the *inv* mutant, argue that YadA does not directly promote uptake into cells overlying the Peyer's patch. In *Y. pseudotuberculosis*, it is likely that the defective translocation of the *inv* mutant is a consequence of YadA binding to intestinal mucus, preventing access of bacteria to the Peyer's patch surface. That we see low levels of wild-type *Y. pseudotuberculosis* binding to mucus indicates that invasin is the dominant adhesin in this strain, and it may interfere with YadA function. It should be noted that in the related *Y. enterocolitica* species, mutations in *yadA* have much more striking defects than seen in *Y. pseudotuberculosis*. YadA is clearly required for maximal virulence in *Y. enterocolitica* (30).

The apparent role of YadA during infection of an animal by *Y. pseudotuberculosis* is to increase the concentration of bacteria within mucus at the Peyer's patch surface, allowing a population that potentially replicates within this site to be established. Once bacteria derived from this population encounter a cell harboring a receptor for invasin, translocation may readily occur. This proposed strategy is more complex than suggested by studies of the interaction of bacteria with cultured mammalian cells. In both the cultured-cell and animal infection models, wild-type organisms require invasin for uptake. During translocation across the intestinal epithelium, in the absence of invasin and YadA, other *Y. pseudotuberculosis* factors can compensate for efficient translocation, or there may be a pathway via M cells that promiscuously internalizes bacteria.

On the basis of this study and that of Pepe and Miller (29), there are at least three pathways that allow enteropathogenic *Yersinia* to translocate across the intestinal epithelium. Two of these pathways, seen in the wild type and the *inv yadA* mutants, result in colonization of the Peyer's patch, whereas the third appears to bypass this site entirely (29). Wild-type bacteria were observed within UEA-1 lectin-reactive epithelial cells overlying Peyer's patches, indicating a tropism of the microorganisms for M cells. Strikingly, these are the only cells in the intestinal epithelial layer that express the β_1 integrin receptors for invasin (24). The invasin-independent pathway promoted by *Y. pseudotuberculosis* in the absence of YadA function also results in colonization of the Peyer's patch. In this case, the ligand-receptor pair involved in translocation is unknown, although presumably the bacteria are internalized by M cells before transit into lymphoid tissue. The final pathway, seen in both *Y. enterocolitica* (29) and *Y. pseudotuberculosis inv* mutants (33), results in lethal systemic infection in the mouse. This route appears to largely bypass colonization of the Peyer's patch via unknown host cells (Fig. 1) (29). Although unproven, it is possible that there are phagocytic cells that have the ability to carry microorganisms from the intestinal lumen into the subepithelial layer via this pathway. Alternatively, microscopic damage to the intestinal epithelial layer may allow a portal of entry for the microorganism. As previously discussed (29), this last pathway may be limited to the model infection systems used for analysis of enteropathogenic *Yersinia*. Human disease rarely results in colonization beyond the mesenteric lymph nodes, so the Peyer's patch-independent pathway leading to systemic disease may not exist in humans.

A variety of serotypes of enteropathogenic *Yersinia* species uniformly express functional versions of YadA and invasin, indicating selective pressure to retain these proteins. The results in this study do not directly explain this selective pressure, but these proteins are likely to be required for human disease. In human populations, enteropathogenic yersinia presumably initiate disease with much lower doses of bacteria and under conditions in which there is heavier competition from the intestinal flora than found in oral models of animal infection. Faced with these prospects, YadA could become a critical factor for colonization of the human mucosal surface, facilitating replication of bacteria in the mucus near Peyer's patches and increasing the effective dose of the pathogen at the epithelial surface. With efficient mucus binding via YadA necessary to increase the population size, *Y. pseudotuberculosis* would require invasin to promote specific translocation into the Peyer's patch, as illustrated in this study. The alternate model, that invasin and YadA are required for systemic disease, is not supported by previous animal studies (33) or by the fact that human systemic diseases caused by these pathogens are rare.

The accepted pathway for systemic infection of the mouse by enteropathogenic *Yersinia* is that the bacteria initially colonize the Peyer's patch and drain into the mesenteric lymph nodes,

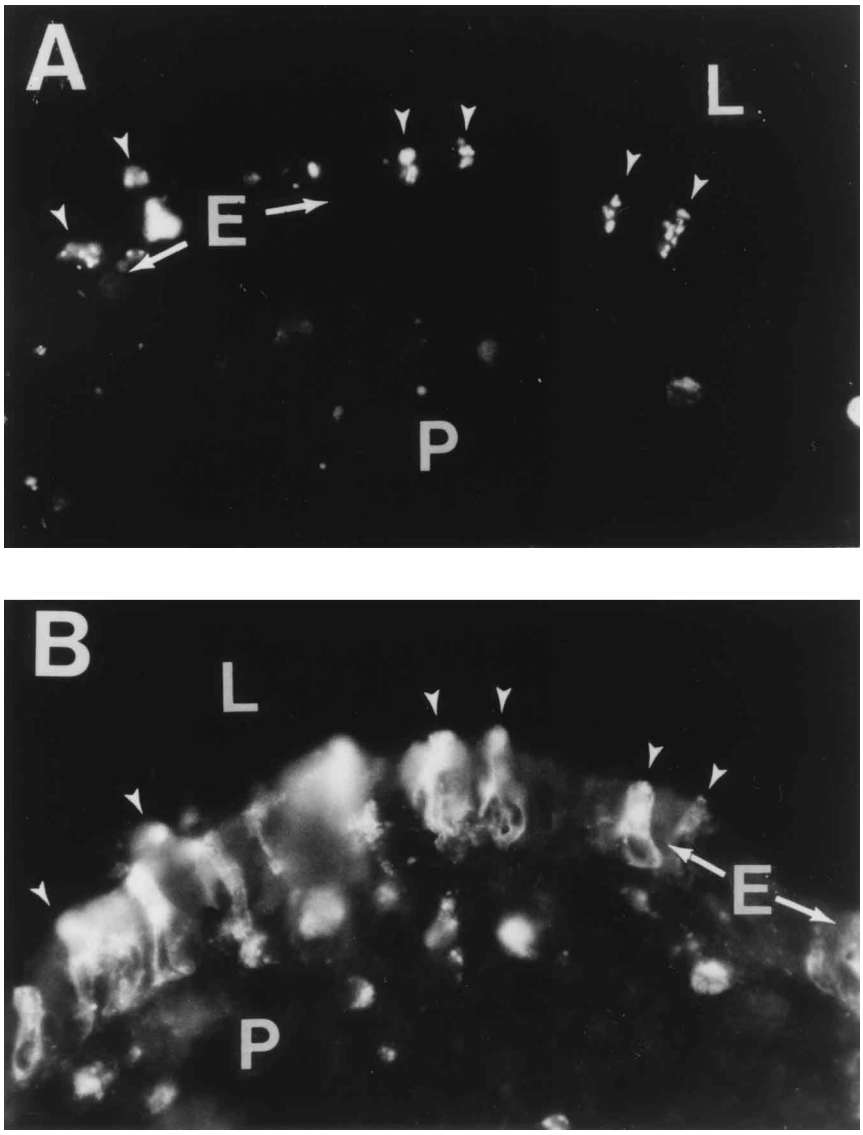


FIG. 7. Infection of mouse ligated loops with a *psaA* mutant of *Y. pseudotuberculosis*. Mouse ligated loops were prepared as described in Materials and Methods and infected with 10^8 TAMRA-labeled bacteria. After 45 min, Peyer's patch tissue was excised and prepared for microscopy. (A) Infection with AM2770 (*psaA*); (B) same field as in panel A, after the tissue was probed with lectin UEA-1 conjugated to FITC. Panel A was viewed under the rhodamine channel, whereas panel B was viewed under the fluorescein channel. E, epithelial layer; L, intestinal lumen; P, Peyer's patch. Bacteria (A) and M cells (B) are indicated (arrowheads).

offering a portal of entry into deeper tissues such as the liver and spleen. This proposed pathway is inconsistent with previous results indicating that systemic murine infection by *inv* mutants can take place in the absence of regional lymph node colonization (29, 33). The picture that emerges from these studies is that there are several routes of spread of enteropathogenic *Yersinia* after initial oral inoculation of the microorganism. There is no proof that initial colonization of one site necessarily leads to colonization of another. In particular, bacteria that serve as the source of the systemic disease are not necessarily derived from small populations established within the Peyer's patch. Analysis of whether bacteria found in the Peyer's patch are clonal with those that ultimately cause systemic disease will be required to determine whether spread is initiated from this site or whether colonization of this site leads to a self-limiting localized disease dependent on the expression of invasin.

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