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Invasin Expression in *Yersinia pseudotuberculosis*

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A 3.2-kb region on the chromosome of *Yersinia pseudotuberculosis*, called *inv*, encodes invasin, a 103-kDa protein of the bacterial outer membrane. Invasin mediates bacterial entry into cultured animal cells. Six *Y. pseudotuberculosis* strains isolated from animal or human infections were analyzed for the presence of *inv*-related sequences with a radiolabeled *inv* clone, pRI203. We found that *inv*-specific sequences were present in all strains studied. Strains cured of virulence plasmid pYV were studied by Western immunoblot analysis with a monoclonal antibody directed against invasin. All but one strain produced invasin, but some strains produced more invasin than others. A strong correlation was found between the level of invasin production by these strains and their ability to enter into HEp-2 or CHO cells. The virulence of these strains was assessed in a murine model by measuring the number of bacteria in the spleen after intravenous challenge or in the mesenteric lymph nodes after intragastric challenge. The capacities of strains to invade cultured mammalian cells and to colonize the spleen were strongly correlative. In contrast, the ability of strains to translocate from the intestinal lumen to the mesenteric lymph nodes after intragastric inoculation did not correlate with their in vitro invasiveness.

Yersinia pseudotuberculosis is a gram-negative bacterium that causes epizootic outbreaks of diseases in birds and mammals. Occasionally, humans can be infected with *Y. pseudotuberculosis* following ingestion of contaminated food or water. When bacterial cells reach the terminal ileum, they cross the intestinal barrier, gain access to the lymphoid tissue, and cause acute mesenteric adenitis (21). By electron microscopy, it has been observed that yersiniae predominantly colonize the Peyer's patches; bacteria adhere to enterocytes and M cells but are taken up only by M cells (5, 6). Three independent cellular penetration pathways have been described for *Y. pseudotuberculosis* and another enteropathogenic *Yersinia* species, *Y. enterocolitica*. Initially, Isberg and Falkow (9) identified a *Y. pseudotuberculosis* chromosomal gene, designated *inv* (for invasion), that encodes a 103-kDa protein called invasin (Inv). Invasin is found in the bacterial outer membrane and promotes both binding to and invasion of epithelial cells in vitro (13). Invasin can directly attach to mammalian cell lines (10) through the recognition of multiple integrins of the β1 family (11). Miller and Falkow (18) also have identified a *Y. enterocolitica* chromosomal gene, called *ail* (for attachment-invasion locus), that allows bacterial binding to cultured mammalian cell lines. Unlike *inv*, *ail* facilitates bacterial entry into a limited number of host cell types, including CHO cells and a cultured human endometrial cell line (20). Sequences homologous to *ail* are also present on the chromosomes of *Y. pseudotuberculosis* and *Y. pestis* (19). The *ail* gene product in *Y. enterocolitica* is a 17-kDa surface protein. However, the mechanism by which *ail* affects bacterial virulence is unclear, since recent data indicate that it also mediates resistance to complement-mediated serum killing (1). The expression of both chromosomal invasion genes is thermally regulated: invasin synthesis is optimal at 28°C, while the *ail* gene product is best expressed at 37°C (12, 17). A third pathway for *Y. pseudotuberculosis* adherence and entry into epithelial cells, mediated by the *Yersinia* virulence plasmid (pYV), has also been described (8). This plasmid, found in all pathogenic *Yersinia* strains (reviewed in references 2 and 3), encodes a set of temperature-inducible proteins (reviewed in reference 4). YadA (formerly called P1 or Yop1), which is exposed on the cell surface, is possibly the adhesin-invasin involved in this third pathway (23).

The most efficient pathway for the entry of *Y. pseudotuberculosis* into cultured mammalian cell is mediated by invasin (7). However, invasin production in this species has only been studied with strain YPIII, in which *inv* was first identified (9). The aim of this work was to analyze *inv* expression in other *Y. pseudotuberculosis* strains and to determine the relevance of the in vitro invasion assay by comparing the ability of these strains to penetrate cultured mammalian cells and to invade tissues in infected mice.

Six strains (strains IP2775, IP2777, and IP2790, belonging to serogroup I, and strains IP2515, IP2666, and IP2126, belonging to serogroups II, III, and V, respectively) isolated in France from human or animal infections were studied. All of the strains, except for strain IP2126, carried plasmid pYV, which was detected by colony hybridization (16) with a radiolabeled probe consisting of a 5.3-kb *Bam*HI fragment of virulence plasmid pIB1 located in the Ca^{2+} dependence locus (24). For this study, all strains were cured of virulence plasmid pYV (denoted by a "c") on MOX medium as previously described (25). We first analyzed these strains for the presence of *inv*-related sequences by colony hybridization with radiolabeled plasmid pRI203, a pBR325 derivative that encompasses the *inv* locus of *Y. pseudotuberculosis* YPIII (13). The plasmid hybridized under high-stringency conditions with colonies of all six strains (data not shown), but several patterns were observed when the *inv* probe was hybridized with *Eco*RV-digested DNA (Fig. 1). Similarly, Miller et al. found two different *inv* hybridization patterns when they studied nine American strains of *Y. pseudotuberculosis* (19).

inv expression in these strains was assessed at 28 and 37°C by Western immunoblot analysis with monoclonal antibody

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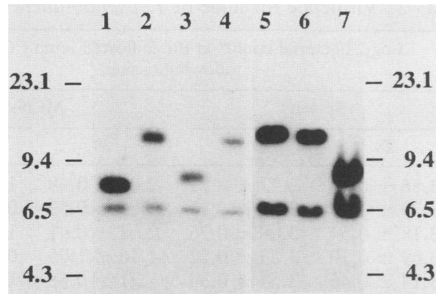

FIG. 1. Autoradiogram of nitrocellulose membranes after hybridization of *Eco*RV-digested *Y. pseudotuberculosis* DNA with ^{32}P-labeled plasmid pRI203. Lanes: 1, strain IP2775; 2, strain IP2777; 3, strain IP2790; 4, strain IP2515; 5, strain YPIII; 6, strain IP2666; 7, strain IP2126. The positions and sizes (in kilobases) of bacteriophage λ DNA digested with *Hin*dIII are indicated to the left and right.

(MAb) 3A2, directed against invasin (15). Two-milliliter quantities of overnight bacterial cultures, adjusted to an A_{620} of 0.5, were pelleted by centrifugation, washed once with phosphate-buffered saline (PBS), and then resuspended in 200 μl of Laemmli solubilization buffer (14). Proteins were fractionated on a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide running gel and then transferred to nitrocellulose membranes. The membranes were incubated with MAb 3A2 and then exposed to a goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate. The antibody binding was visualized by incubation of the membranes with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Invasin production by the six test strains and the Inv^{+} and Inv^{-} control strains (respectively, strain YPIIIc and its derivative strain YP202, resulting from the insertion of a *kan* fragment into *inv* [13]) is depicted in Fig. 2. Multiple protein species reacted with MAb 3A2, as has been observed already with another MAb directed against invasin (11); this result was most likely due to the fact that the MAb recognizes not only the 103-kDa invasin protein (highest band) but also its degradation and premature termination products (13). Invasin was detected in all strains tested, except for strain IP2126. Four of the strains (IP2775c, IP2777c, IP2790c, and IP2515c), however, qualitatively produced much more invasin than the other two strains (IP2666c and YPIIIc). Moreover, *inv* expression was higher at 28°C than at 37°C for the former four strains, while the latter two strains showed low *inv* expression at both temperatures. Finally, no correlation was found between the level of *inv* expression and the *inv* hybridization pattern.

To determine what effect, if any, the level of Inv production has on *Yersinia* invasiveness, we assessed the entry of these six strains into two different mammalian cell lines. Human laryngeal epithelial (HEp-2) or Chinese hamster ovary (CHO) cells seeded into 24-well plates at a density per well of 10^5 cells in RPMI 1640 (for HEp-2 cells) or Eagle (for CHO cells) medium containing 5% fetal bovine serum were grown overnight at 37°C in a 5% CO_2 atmosphere. Approximately 3×10^6 to 6×10^6 bacteria from an overnight bacterial culture grown at either 28 or 37°C were added to the tissue culture monolayers. Bacteria were centrifuged onto the monolayers at $500 \times g$ for 10 min to synchronize the time of infection. Infected monolayers were incubated at 37°C in a 5% CO_2 atmosphere for 90 min. Following the infection step, the medium was removed and the monolayers were washed three times with PBS to eliminate nonadherent bacteria. Titers of internalized bacteria were determined by reincubating infected monolayers for 90 min at 37°C with fresh tissue culture medium containing 100 μg of gentamicin per ml, an antibiotic that kills extracellular but not intracellular bacteria (8). The medium was then removed, and the monolayers were washed three times to eliminate gentamicin. No cytotoxic effect was observed by microscopic examination of infected cells. Internalized bacteria were released from the monolayers by adding 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) and measuring viable counts on agar plates. The entry of *Y. pseudotuberculosis* into HEp-2 and CHO cells was expressed as follows: percent invasion = (number of bacteria resistant to gentamicin/number of bacteria added) × 100. As shown in Table 1, the most invasive strains were those producing high levels of invasin. In addition, such strains pregrown at 28°C entered the mammalian cells much more efficiently than the same strains pregrown at 37°C. In contrast, not only were the strains producing low levels of invasin less invasive than the strains producing high levels of invasin, but also their invasiveness was not strikingly different when they were cultured at 28 versus 37°C. Finally, strain IP2126, which did not produce a detectable level of invasin, did not invade

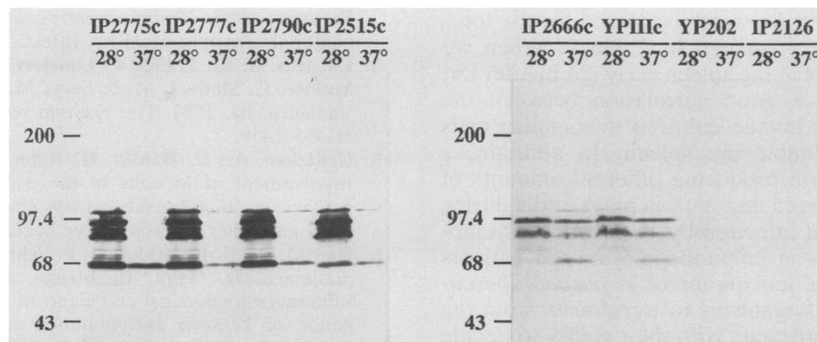

FIG. 2. Western immunoblot analysis of *inv* expression in *Y. pseudotuberculosis* strains. Bacteria were grown overnight at 28 or 37°C in Luria-Bertani broth, and bacterial suspensions were adjusted to an A_{620} of 0.5. Cells were pelleted, washed, and resuspended in Laemmli buffer. After 30 min of incubation at room temperature, a 10-μl volume of material was fractionated on an SDS–7.5% polyacrylamide gel, transferred to nitrocellulose membranes, and probed with MAb 3A2 (diluted 1:1,500). Strains YPIIIc (Inv^{+}) and YP202 (Inv^{-}) were used as controls for invasin expression. The positions and sizes (in kilodaltons) of protein standards are indicated on the left.

TABLE 1. Entry of *Y. pseudotuberculosis* into mammalian cell lines

Strain	% Invasion[a] at the indicated temp (°C) of cell line:			
	HEp-2		CHO	
	28	37	28	37
IP2775c	18.6 ± 1.8	1.3 ± 0.1	21.9 ± 3.5	2.1 ± 0.2
IP2777c	30.8 ± 3.1	0.61 ± 0.07	13.9 ± 1.7	2.2 ± 0.2
IP2790c	25.7 ± 4.8	1.6 ± 0.1	41.0 ± 3.8	3.3 ± 0.8
IP2515c	26.8 ± 1.0	0.47 ± 0.19	14.0 ± 1.8	2.0 ± 0.2
IP2666c	0.59 ± 0.09	0.21 ± 0.06	0.62 ± 0.06	2.4 ± 0.4
YPIIIc	5.3 ± 1.3	0.28 ± 0.09	2.5 ± 0.4	1.0 ± 0.1
YP202	0.008 ± 0.004	0.014 ± 0.003	0.18 ± 0.02	0.47 ± 0.09
IP2126	0.002 ± 0.001	0.009 ± 0.003	0.13 ± 0.05	0.47 ± 0.10

[a] Mean ± standard deviation for triplicate assays.

TABLE 2. Virulence for mice of *Y. pseudotuberculosis*

Strain	Log_{10} bacterial count[a] at the indicated temp (°C) in the following tissue:			
	Spleen[b]		MLNs[c]	
	28	37	28	37
IP2775c	3.56 ± 0.13	2.73 ± 0.29	2.01 ± 0.49	1.24 ± 0.45
IP2777c	3.79 ± 0.20	3.01 ± 0.15	1.69 ± 0.52	0.53 ± 0.39
IP2790c	3.18 ± 0.54	3.08 ± 0.26	2.02 ± 0.41	1.77 ± 0.56
IP2515c	3.69 ± 0.10	3.23 ± 0.22	1.46 ± 1.06	0.63 ± 0.79
IP2666c	3.14 ± 0.46	3.54 ± 0.30	1.21 ± 0.68	1.23 ± 0.65
YPIIIc	2.56 ± 0.21	2.15 ± 0.17	1.41 ± 0.60	0.73 ± 0.73
YP202	≤2.00	2.30 ± 0.24	0.27 ± 0.38	0.18 ± 0.26
IP2126	2.07 ± 0.15	2.12 ± 0.24	2.32 ± 1.07	0.83 ± 0.64

[a] Mean ± standard deviation for four or five mice.

[b] Swiss mice were inoculated i.v. with $\sim 5 \times 10^3$ pYV-negative bacteria grown at 28 or 37°C. Twenty-four hours later, yersiniae were counted in the spleen.

[c] DBA-2 mice were given i.g. $\sim 3 \times 10^{10}$ pYV-negative bacteria grown at 28 or 37°C. Six hours later, yersiniae were counted in the MLNs.

eukaryotic cells, and neither did *inv*-deficient strain YP202. These results clearly show a strong correlation between the level of invasin production by these strains grown at 28°C and their ability to enter HEp-2 or CHO cells. At 37°C, the results were still correlated with invasin levels but were difficult to interpret because of *ail* expression.

We also investigated the virulence for mice of the six pYV-negative strains and control strains YPIIIc (Inv^+) and YP202 (Inv^-). We studied both the colonization of the spleen following intravenous (i.v.) inoculation of bacteria and the passage of bacteria from the intestinal lumen to the mesenteric lymph nodes (MLNs), i.e., translocation, after intragastric (i.g.) inoculation of bacteria. Microorganisms were counted in MLNs early (6 h) after the infectious challenge to account for only bacterial translocation and not bacterial growth in this tissue. Six- to 8-week-old Swiss or DBA-2 female mice (Charles River Breeding Laboratories, Wilmington, Mass.) were given *Y. pseudotuberculosis* by either the i.v. or the i.g. route. Challenge bacterial were prepared from overnight cultures grown in Luria-Bertani broth at either 28 or 37°C. Bacteria were washed once and resuspended in either distilled water or PBS. Groups of four or five mice were given $\sim 5 \times 10^3$ bacteria i.v. (in a volume of 0.5 ml) or $\sim 3 \times 10^{10}$ bacteria i.g. (in a volume of 1 ml) by use of a gastric tube. Infected mice were sacrificed with chloroform anesthesia, and their spleens and MLNs were removed aseptically and homogenized in PBS. Homogenates were spread on agar, and colonies were counted after incubation for 48 h at 28°C. Minimal detection limits were 1 *Y. pseudotuberculosis* cell MLN^{-1} and 100 *Y. pseudotuberculosis* cell $spleen^{-1}$. Results were expressed as the log_{10} bacterial counts and are presented in Table 2. When we measured bacterial counts in the spleen early (24 h) after i.v. challenge, we observed a good correlation between the ability of the bacteria to invade cultured mammalian cells and their ability to colonize the spleen. In addition, a virulence assay of bacteria producing different amounts of invasin in mice also indicated that invasin plays a role during the hematogenous step of infection by *Y. pseudotuberculosis*, which mainly occurs in immunocompromised patients (21). In contrast, after i.g. inoculation of *Y. pseudotuberculosis*, the ability of microorganisms to translocate from the intestinal lumen did not correlate with their ability to invade HEp-2 and CHO cells. This discrepancy might be explained by a difference in *inv* expression in vitro and in the intestinal lumen, as has been demonstrated already for pYV genes (22). It must also be noted that the nature of cells used for in vitro invasion assays and the nature of those involved in crossing the intestinal mucosa are different. HEp-2 and CHO cells are nonprofessional phagocytic cells, whereas M cells, which transport yersiniae from the intestinal lumen to the lamina propria (5), are phagocytic cells. M cells carry virtually any attached particle, and theoretically one would expect no requirement of invasin for the internalization of *Y. pseudotuberculosis* into these cells. Although speculative, the reduced (10-fold lower) translocation of strain YP202 (Inv^-) compared with that of the parent strain, YPIIIc (Inv^+), that we observed (Table 2) could have been due merely to decreased bacterial binding to M cells. That an *inv*-deficient strain still translocated also suggests that another chromosomal factor(s) is involved in crossing of the intestinal barrier.

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