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# Invasin Production by *Yersinia pestis* Is Abolished by Insertion of an IS*200*-Like Element within the *inv* Gene

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**The two enteropathogens *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* penetrate eukaryotic cells in vitro through invasin, a surface-exposed protein. In contrast, *Yersinia pestis*, the causative agent of plague, is unable to enter mammalian cell lines, although the *inv* gene is present on its chromosome. Although 99.3% identical to the *inv* gene of *Y. pseudotuberculosis*, the *Y. pestis inv* gene was disrupted in its central region by a 708-bp IS*200*-like element. Multiple copies of this insertion sequence element were found within the genome of the plague bacillus.**

Plague is a highly contagious disease that persists endemically in many countries in the world, with unpredictable epidemic resurgences. The causative agent is *Yersinia pestis*, a nonmotile, capsulated, gram-negative bacterium transmitted to humans and susceptible animals through flea bites or aerosols (5). Virulence of *Y. pestis* is due to its capacity to overwhelm the defense mechanisms of infected hosts, resulting in a rapid extracellular multiplication of bacteria in host tissues (38). *Y. pestis* harbors three plasmids that are required for the expression of virulence (7). The smallest, 9.5-kb plasmid (pPst) encodes the outer membrane protein Pla (plasminogen activator), a protease interfering with the blood coagulation and complement activation pathways (37). The 70-kb plasmid, called pYV (plasmid *Yersinia* virulence), harbors genes that encode released proteins, the Yops (*Yersinia* outer membrane proteins), of which YopH and YopE inhibit phagocytosis (reviewed in reference 39). Finally, the largest, 100-kb plasmid, designated pFra, is associated with the synthesis of a capsular antigen (fraction 1) and a murine toxin (30).

*Y. pestis* is an extracellular pathogen unable to invade epithelial cells in vitro (31, 34). In contrast, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, which cause self-limiting mesenteric lymphadenitis and ileitis (5), are invasive for mammalian cell lines (15). The ability of bacteria to invade cells in vitro is mainly due to the product of a chromosomal gene called *inv*, which is highly homologous between the two species (16, 18, 23, 40). The *inv* gene encodes invasin, an outer membrane protein of 92 and 103 kDa in *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. Isberg and Leong (17) have demonstrated that invasin directly attaches to mammalian cells through multiple integrins and induces bacterial internalization inside eukaryotic cells. The biological activity of invasin is located in the last 192 amino acids of its C terminus. On the basis of oral infection of animals with *inv*-deficient bacteria, it is believed that invasin may be necessary for microorganisms to cross the intestinal mucosa (28, 35). Surprisingly, the presence of *inv*-related sequences was also demonstrated in *Y. pestis* by Southern blot analysis (24), but the meaning of this finding is not yet understood.

To amplify the *inv* gene from *Y. pestis*, purified DNA extracted from pYV-cured strain 6/69Mc as previously described (22) was used as template material in PCRs with three sets of primers (Fig. 1). Oligonucleotide primers were synthetized (Bioprobe, Montreuil, France) on the basis of the published *inv* sequences from *Y. pseudotuberculosis* and *Y. enterocolitica* (18, 40). The mixture contained 100 μg of template DNA, 3 pM primers, 0.04 mM each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP; Pharmacia, Uppsala, Sweden), 5 U of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, and 0.1% Triton X-100 in a total volume of 100 μl. Thirty cycles were performed, each consisting of a denaturation step (1 min at 94°C, first cycle for 5 min), annealing step (1 min at 55°C), and an extension step (1.5 min at 72°C). After a final extension (10 min at 72°C), the reaction was cooled at 4°C and 5 μl was analyzed by electrophoresis on a 0.8% agarose gel. Amplification reactions yielded, both from *Y. pestis* 6/69Mc DNA and plasmid pRI203 harboring the *inv* gene from *Y. pseudotuberculosis* (18), a fragment of ~0.6 kb with primers 1f and 1r and a fragment of ~1.6 kb with primers 3f and 3r. With primers 2f and 2r, a fragment of ~1.7 kb was amplified from *Y. pestis*, whereas a product of ~0.9 kb was obtained from plasmid pRI203. Amplification products from *Y. pestis* were inserted into phage M13 mp18 and mp19 (Boehringer Mannheim GmbH, Mannheim, Germany) as previously described (32).

The nucleotide sequence of the *inv* gene from *Y. pestis* was determined by the dideoxynucleotide chain termination method (33), using T7 DNA polymerase (Sequenase version 2.0) and a sequencing kit (U.S. Biochemical, Cleveland, Ohio). This gene was disrupted in the central region (position 982) by an unrelated 708-bp nucleotide sequence (Fig. 1). Comparison of the central 708-bp sequence with the GenBank and EMBL database revealed a high identity (85%) with insertion element IS*200* identified in *Salmonella* and *Escherichia coli* isolates (2, 12, 19). By contrast, there was no significant homology with the IS*100* element which has been recently found in multiple copies within *Y. pestis* (8, 9, 29). Consequently, *inv* was split into two open reading frames (ORFs) of 1,059 and 2,070 bp, respectively. The 5′ ORF possessed the transcriptional and translational signals of *inv* and ended after 50 codons within the insertion sequence (IS). The 3′ ORF started with six codons within the IS but did not contain any start codon preceded by a putative ribosome-binding site; therefore, we assumed that the 3′ ORF is not expressed in *Y. pestis*. The nucleotide sequence of *Y. pestis inv* was 99.3 and 54% identical to *Y. pseudotuberculosis* and *Y. enterocolitica inv* sequences, respectively, and identities at the protein level were 99.1 and 63.5%.

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FIG. 1. Nucleotide sequence of the *inv* gene from *Y. pestis* 6/69M. Synthetic oligonucleotides (forward, f; reverse, r) used in PCR-based amplification of *inv* nucleotide sequences are indicated by arrows above the nucleotide sequence. The nucleotide sequence of the IS is shown in lowercase. Asterisks mark the stop codons.

Computer analysis of the nucleotide sequence of the IS*200*-like element revealed an ORF that started at position 1124 and spanned almost the entire length of the element. It encoded a putative protein of 152 amino acids strongly homologous to the putative proteins encoded by other IS*200* elements (Fig. 2). Like IS*200* (3), the IS did not contain any inverted repeats, which is unusual for a transposable element (11). In addition, the G+C content of this IS was 46.1 mol%. A striking homol-

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Y. pestis          MRDEKSLAHTRWNCKYHIVFAPKYRRQVFYREKRRAIGSILRKLCEWKNVNI----------
E. coli            MGNEKSLAHTRWNCKYHIVFAPKYRRQVFYREKRRATGSILRKLCEWKSVRI----------
S. typhimurium     MGDEKSLAHTRWNCKYHIVFAPKYRRQAFYGEKRRAVGSILRKLCEWKNVRIPGSRMLCRSYSHASGDP
                   * .********************** ******** ***********.* *

Y. pestis          LEAEYCVDH---IHMLLEIPPKMSVSGFMGYLKGKSSLMLYEQFGDLKFKYRNREFWCRGYYVDTVGKNT
E. coli            LEAECCADH---IHMLVEIPPKMSVSGFMGYLKGKSSLMLYEQFGDLKFKYRNRDFWCRGDYVDTVDKNT
S. typhimurium     AEDECVEFHGISEGMLLEIPPKMSVSSFMGYLKGKSSLMLYEQFGDLKFKYRNREFWCRGYYVDTVGKNT
                    * *    *     **.********* ****************************.***** ***** ***

Y. pestis          ARIQEYIKHQLEEDKMGEQLSIPYPGSPFTGRKX
E. coli            AKIQDYIKHQLEEDKMGEQLSIPYPGSPFTGRKX
S. typhimurium     AKIQDYIKHQLEEDKMGEQLSIPYPGSPIYGPX-
                   *.**.********************** *
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FIG. 2. Alignment of the amino acid sequence deduced from the nucleotide sequence of IS*200* from *Y. pestis* 6/69M, *E. coli* EcoR8, and *S. typhimurium* LT2. Asterisks and points indicate identical and similar amino acids, respectively. Dashes are gaps introduced to optimize homology between the sequences.

ogy has been previously reported between three genes belonging to the invasion locus of *Salmonella typhimurium* and three pYV genes from pathogenic *Yersinia* species (10, 13). The G+C content of these *Salmonella* invasion genes was estimated to be 45 mol%, similar to that for *Yersinia* species (45 mol%) and different from that for *Salmonella* species (estimated to be 52 mol%). These data reinforce the hypothesis proposed by others (10, 13) that gene exchanges, including invasion genes and IS, can occur between *Yersinia* and *Salmonella* species. This phenomenon may be related to the similar ecologic niches of these microorganisms.

Similarly, we analyzed the *inv* genes from four other *Y. pestis* strains (12, PKR XXIV, Th, and 55-1239, kindly provided by E. Carniel, Centre National de Référence des *Yersinia*, Institut Pasteur, Paris, France). They were isolated between 1944 and 1955 in distant geographical areas throughout the world and belong to different ribotypes as defined by Guiyoule et al. (14). Using primers 2f and 2r with PCR, we amplified a ~1.7-kb fragment from the DNA of each isolate. After inserting the amplified DNA fragments into the M13 vector, we sequenced the IS and its surrounding *inv* region. For each of the four strains studied, we found that first, the nucleotide sequence of the IS was identical to that from strain 6/69Mc, and second, the IS was always inserted at the same site within the *inv* gene, indicating the occurrence of a stable insertional and early event during the evolution of this pathogen. However, this site does not seem to be a hot spot because an identical site is present within the *inv* gene of *Y. pseudotuberculosis* without any insertion of IS*200* even though multiple copies of the insertion element have been found in the genome of this species (see below).

The nucleotide sequence data suggested that the inability of *Y. pestis* to invade mammalian cell lines might be due to the insertion of an IS*200*-like element within the *inv* gene. To test this hypothesis, plasmid pRI203 harboring the *inv* gene and its own promoter from *Y. pseudotuberculosis* was introduced into *Y. pestis* 6/69Mc by electroporation, using the protocol developed for *E. coli* by Dower et al. (6). Invasin production was assessed in recombinant *Y. pestis* by Western blot (immunoblot) analysis with a monoclonal antibody (MAb 3A2, kindly provided by Ralph Isberg, Tufts University, Boston, Mass.) directed against the last 192 amino acids of invasin (Fig. 3A). This specific MAb recognized several bands in *Y. pseudotuberculosis* IP2790c and no bands in *Y. pestis* 6/69Mc. *Y. pestis*, complemented in *trans* with the *inv* gene from *Y. pseudotuberculosis*, produced invasin. The invasiveness of recombinant *Y. pestis* was then studied in vitro on HEp-2 cells, as described previously (35), using the parental strain 6/69Mc (pYV cured) and the *Y. pseudotuberculosis* invasive strain IP2790c (pYV cured) as controls (Fig. 3B). Expression of invasin by *Y. pestis* increased its ability to enter into HEp-2 cells, compared with the failure of the parental strain to penetrate cells (Fig. 3B). However, the level of invasiveness was 100-fold lower than for *Y. pseudotuberculosis* strains, as seen in strain IP2790c (Fig. 3B) or recombinant *E. coli* producing invasin (20).

The presence of additional copies of an IS*200*-like element in *Y. pestis* was investigated by Southern blot analysis. Total DNA from strains 6/69Mc, PKRXXIV, Th, 12, and 55-1239 was digested with *Hin*cII (New England Biolabs, Beverly, Mass.), a restriction endonuclease that does not cut within the IS. Digested DNA fragments were hybridized (35) with a 421-bp DNA probe internal to the IS*200*-like element. As observed in *Salmonella* species (12), multiple copies of the IS*200*-like element were present in the genome of *Y. pestis* (Fig. 4A). The exact number of IS copies present in the bacterial genome was probably underestimated, because bands

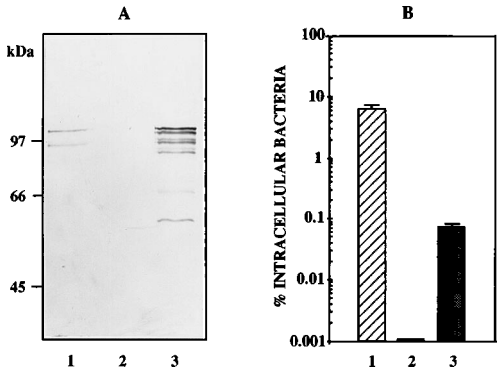


FIG. 3. *inv* expression by *Y. pestis*. (A) Western blot analysis of invasin production. Bacteria were grown overnight in LB broth, and bacterial suspensions were adjusted to an $A_{620}$ of 0.5. Cells were pelleted, washed, and suspended in Laemmli buffer. A 10-μl volume of material was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel, transferred to a nitrocellulose sheet, and incubated with a MAb specific for invasin (MAb 3A2). (B) Bacterial entry into HEp-2 cells. Invasiveness was expressed as follows: percent invasion = (number of bacteria resistant to gentamicin/number of bacteria added to cell monolayers) × 100. Columns: 1, *Y. pseudotuberculosis* IP2790c used as a control for invasin expression; 2, *Y. pestis* 6/69Mc; 3, *Y. pestis* 6/69Mc(pRI203).

giving a strong hybridization signal may have corresponded to multiple restriction fragments of similar sizes. We are currently characterizing the DNA regions (other than *inv*) where the IS*200*-like element has inserted into the genome of *Y. pestis*. Furthermore, we do not know yet whether the IS is carried on virulence plasmids. The five strains of *Y. pestis*, isolated from distant periods of time and geographical areas, exhibited distinguishable IS fingerprintings, as shown by Southern blot analysis (Fig. 4). Interestingly, strains 6/69Mc and Th, which belong to the same biotype (*orientalis*) and ribotype (B) (14), displayed different hybridization patterns. Thus, the IS*200*-like

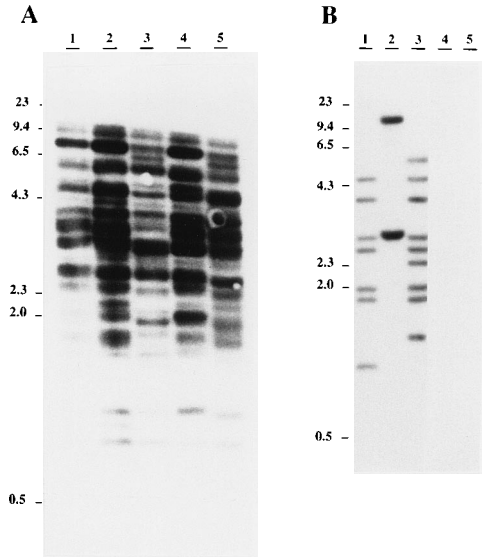


FIG. 4. IS*200* hybridization to genomic DNA of pathogenic yersiniae. Autoradiograms of a nylon membrane after hybridization of *Hin*cII-digested DNA with an internal fragment of IS*200* labeled with [$^{32}$P]dCTP. (A) *Y. pestis* 6/69Mc (lane 1), 12 (lane 2), PKR XXIV (lane 3), Th (lane 4), and 55-1239 (lane 5); (B) *Y. pseudotuberculosis* IP2775 (lane 1), IP2790 (lane 2), and IP2777 (lane 3) and *Y. enterocolitica* IP383 (lane 4) and IP864 (lane 5). The positions and sizes (in kilobases) of bacteriophage lambda DNA digested with *Hin*dIII are indicated to the left.

element may be a useful tool for plague epidemiology. Similarly, the presence of IS*200*-like element was studied in *Y. pseudotuberculosis* serotype 1 strains (strains IP2775, IP2777, and IP2790c from the Centre National de Référence des *Yersinia*) and *Y. enterocolitica* serotype O:3 and O:9 strains (strains IP383 and IP864, respectively, from the Centre National de Référence des *Yersinia*). The IS was detected in all *Y. pseudotuberculosis* strains tested (two to nine copies, depending on the strain), whereas IS*200* was not found in *Y. enterocolitica* (Fig. 4B).

The biological meaning of the abrogation of invasin synthesis in *Y. pestis* by an IS is not yet understood. In fact, *Y. pestis* is an extracellular pathogen that does not seem to require invasion factors during its infectious process and its cycle of transmission. Although this pathogen can be transmitted by aerosols and even by the oral route in carnivores and rodents (5), it is mainly propagated by fleas that transcutaneously inoculate bacteria through bites. Moreover, in these vectors, bacteria are not invasive and remain confined within the foregut, the proventriculus (5). In addition to invasin, another invasion factor, YadA, is not expressed in *Y. pestis*, although the gene is present in this pathogen. YadA is a pYV-encoded protein involved in the in vitro invasion of mammalian cell lines by enteropathogenic *Yersinia* species (reviewed in reference 39). In *Y. pestis*, the *yadA* gene contains a point mutation leading to a reading frame shift (31, 36). From DNA-DNA hybridization, it is known that *Y. pestis* and *Y. pseudotuberculosis* belong to the same species, having more than 90% DNA identity (1). The near identity of the *inv* gene of *Y. pestis* with that of *Y. pseudotuberculosis* is consistent with this finding. *Y. pseudotuberculosis* is widely spread in nature, with a saprophytic life associated with motility, invasiveness, and expression of multiple metabolic pathways, including urea and sugar catabolism and amino acid biosynthesis (4). In contrast, *Y. pestis* is a strict pathogen mainly infecting rodents and surviving only in the environment, without a saprophytic life (25). It is a nonmotile, capsulated, noninvasive extracellular bacterium that is devoid of several metabolic pathways found in *Y. pseudotuberculosis* (4). It is tempting to speculate that *Y. pestis* may have derived from *Y. pseudotuberculosis* to become a strict highly virulent pathogen. This adaptation may have resulted from the loss of the expression of genes not required for its parasitism, including invasion, motility, and some metabolic genes, and from the acquisition of plasmid-borne virulence genes encoding the capsular antigen, toxins, and proteases, favoring its extracellular life in host tissues.

**Nucleotide sequence accession number.** The sequence shown in Fig. 1 has been submitted to the GenBank nucleotide sequence database under accession number U22457.

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