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The 102-Kilobase Unstable Region of *Yersinia pestis* Comprises a High-Pathogenicity Island Linked to a Pigmentation Segment Which Undergoes Internal Rearrangement

CARMEN BUCHRIESER,[1] MICHAEL PRENTICE,[1,2] AND ELISABETH CARNIEL[1]*

Unité de Bactériologie Moléculaire et Médicale, Laboratoire des Yersinia, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Department of Medical Microbiology, St. Bartholomew's and The Royal London School of Medicine and Dentistry, London EC1A 7BE, United Kingdom[2]

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Several pathogenicity islands have recently been identified in different bacterial species, including a high-pathogenicity island (HPI) in *Yersinia enterocolitica* 1B. In *Y. pestis*, a 102-kb chromosomal fragment (*pgm* locus) that carries genes involved in iron acquisition and colony pigmentation can be deleted en bloc. In this study, characterization and mapping of the 102-kb region of *Y. pestis* 6/69 were performed to determine if this unstable region is a pathogenicity island. We found that the 102-kb region of *Y. pestis* is composed of two clearly distinct regions: an ≈35-kb iron acquisition segment, which is an HPI per se, linked to an ≈68-kb pigmentation segment. This linkage was preserved in all of the *Y. pestis* strains studied. However, several nonpigmented *Y. pestis* strains harboring an *irp2* gene have been previously identified, suggesting that the pigmentation segment is independently mobile. Comparison of the physical map of the 102-kb region of these strains with that of strain 6/69 and complementation experiments were carried out to determine the genetic basis of this phenomenon. We demonstrate that several different mechanisms involving mutations and various-size deletions are responsible for the nonpigmented phenotype in the nine strains studied. However, no deletion corresponded exactly to the pigmentation segment. The 102-kb region of *Y. pestis* is an evolutionarily stable linkage of an HPI with a pigmentation segment in a region of the chromosome prone to rearrangement in vitro.

The term pathogenicity island was coined by Hacker et al. to describe two large, unstable DNA regions of the chromosome of uropathogenic *Escherichia coli* (23). This term refers to a usually large (≥35-kb) chromosomal segment that carries genes involved in pathogenicity. Characteristically, pathogenicity islands are bordered on one side by a tRNA gene and, less frequently, may be flanked by insertion sequences. These islands are often unstable, and their deletion occurs at frequencies of 10^{-4} to 10^{-5}. Their GC content is usually different from that of the rest of the host chromosome, suggesting that they originate from horizontal transfer between different bacterial genera (24). The number of gram-negative bacterial species shown to harbor pathogenicity islands has grown steadily and includes uropathogenic (3, 28, 48) and enteropathogenic (33) *E. coli*, *Helicobacter pylori* (9), *Salmonella typhimurium* (35, 44), *Dichelobacter nodosus* (10), and *Vibrio cholerae* (29).

Pathogenicity islands have also been identified in the genus *Yersinia*. In *Yersinia enterocolitica*, such an island is found in high-pathogenicity strains of biotype 1B only and not in low-pathogenicity strains (5). Since the presence of this region determines the level of pathogenicity, it was termed a high-pathogenicity island (HPI). The HPI of *Y. enterocolitica* Ye8081 is 45 kb long and is bordered on one side by an *asn* tRNA gene. It also carries a single copy of four different sequences repeated elsewhere in the chromosome (RS.3, RS.4, IS*1400* [5], and IS*1328* [41]) and genes involved in siderophore-mediated iron acquisition: the yersiniabactin biosynthetic genes *irp2* and, probably, *irp1* (5) and the *fyuA* locus, which codes for the yersiniabactin receptor (42).

In *Y. pestis*, a 102-kb region called the *pgm* locus was first identified by Fetherston et al. (17). This region is deleted en bloc at a frequency of 10^{-5}, probably by homologous recombination between its two flanking IS*100* copies (39). The 102-kb region can be divided into two functionally and physically distinct parts. One carries the *hms* (for hemin storage) locus (36, 38), which confers a pigmented phenotype on colonies grown on Congo red-agar plates and enhances transmission of the microorganism by its flea vector (26). This region has been designated the pigmentation segment in this study. The other region carries the same *irp2*, *irp1*, and *psn*/*fyuA* genes involved in iron acquisition as those of the *Y. enterocolitica* HPI (25), the recently identified siderophore biosynthetic loci *ybtT* and *ybtE* (1), and the activator gene *ybtA* (14). This region is called here the iron acquisition segment.

We wished to determine whether the whole 102-kb region is a true pathogenicity island. Both segments of this region carry genes important for either virulence or disease transmission, and they are deleted en bloc, suggesting that the whole 102-kb region forms a pathogenicity island (5). However, a previous analysis of 43 *Y. pestis* strains revealed that 16% of these strains harbored the *irp2* gene (located on the iron acquisition segment) but were nonpigmented (27). Fetherston et al. also identified a single strain of this type (M23) out of 43 examined (17) and demonstrated that the M23 mutants resulted from different genetic alterations such as a mutation in the *hmsR* gene and at least two different-size deletions (17, 31). To determine whether internal deletion of the pigmentation segment could occur independently from that of the iron acquisition segment, the 102-kb region of strain 6/69 was characterized and used to analyze the different mutants.

* Corresponding author. Mailing address: Institut Pasteur, Unité de Bactériologie Moléculaire et Médicale, Laboratoire des *Yersinia*, 28, rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (33-1)-45-68-83-26. Fax: (33-1)-40-61-30-01. E-mail: carniel2@pasteur.fr.

TABLE 1. Characteristics of the *Y. pestis* strains used in this study

Strain	Abbreviation	Biovar	Geographical origin	Type[a]	
				Pgm	Irp2
6/69	6/69	Orientalis	Madagascar	+	+
6/69$^-$	6/69$^-$	Orientalis	Madagascar	−	−
Turquie 10/1	T10/1	Orientalis	Turkey	−	+
Turquie 10/3	T10/3	Orientalis	Turkey	−	+
Kenya 129	K129	Antiqua	Kenya	−	+
Kenya 164	K164	Antiqua	Kenya	−	+
Kenya 169	K169	Antiqua	Kenya	+	+
Kenya 169.1	K169.1	Antiqua	Kenya	−	+
Hambourg 19	H19	Orientalis	Germany	−	+
Saigon 55-797	S55-797	Orientalis	Vietnam	−	+
Saigon 55-1239	S55-1239	Orientalis	Vietnam	+	+
Saigon 55-1239.1	S55-1239.1	Orientalis	Vietnam	−	+
Congo Belge Lita	CBL	Antiqua	Zaire	−	+

[a] Irp2, presence (+) or absence (−) of the *irp2* gene. Pgm, pigmented (+) or nonpigmented (−) phenotype on Congo red-agar plates.

We show that the 102-kb region of *Y. pestis* is composed of a ca. 35-kb HPI homologous to the HPI of *Y. enterocolitica* and of a ca. 68-kb adjacent pigmentation segment. We also demonstrate that the Pgm$^-$ Irp2$^+$ type observed in nine *Y. pestis* isolates results from several distinct phenomena such as variable deletions involving the pigmentation segment, point mutation in the *hms* locus itself, or mutation in a gene outside the *hms* locus but contributing to the pigmented phenotype. Finally, we show that despite the various rearrangements observed in vitro, the 102-kb region is highly conserved among *Y. pestis* strains of different geographical origins and biotypes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The characteristics of the *Y. pestis* strains used in this study are listed in Table 1. Strain 6/69$^-$ is a derivative of strain 6/69 that has spontaneously lost the *irp2* gene and the surrounding region (7). The geographical origin of the strain corresponds to the name of the country or city in use at the time the strain was isolated. The other *Yersinia* strains used in this study were *Y. pseudotuberculosis* IP32954 (serotype I) and IP32938 (serotype III); *Y. enterocolitica* Ye 8081 (biotype 1B, serotype O:8), IP864 (biotype 4, serotype O:3), and IP383 (biotype 2, serotype O:9); *Y. mollaretii* IP21081; *Y. bercovieri* IP21531; *Y. intermedia* IP21356; *Y. kristensenii* IP21577; and *Y. frederiksenii* IP21689. The *E. coli* strains used were DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), LE392 [*hsdR574 supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*), and XL1Blue {*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac* [F′ *proAB*, *lacI*^{q}ZDM15 Tn*10* (Tet^r)]}. *Yersinia* strains were grown for 24 h (peptone broth) or 48 h (Trypticase soy agar plates) at 28°C. *E. coli* strains were grown at 37°C for 24 h. The pigmentation phenotypes of the *Y. pestis* isolates were determined on Congo red-agar plates after 4 days of growth at 26°C, as described by Surgalla and Beesley (47).

DNA techniques and cloning methods. Isolation and digestion of genomic DNA were performed as previously described (8). Plasmid extractions were done by the method of Birnboim and Doly (2). For PCR-amplified DNA, the products of the reactions were purified by elution from an agarose gel with the Geneclean kit (Bio 101, Inc., La Jolla, Calif.) or by using the Wizard PCR preps DNA purification system (Promega). Double-stranded DNA labeling was performed either radioactively with [^{32}P]dATP (Amersham) or nonradioactively by the enhanced-chemiluminescence reaction (ECL; Amersham) or the digoxigenin (DIG) random primed labeling system (Boehringer). Single-stranded oligonucleotides were labeled with either the 3′ oligonucleotide labeling system (ECL) from Amersham or the DIG-oligonucleotide tailing kit from Boehringer. To screen the cosmid library, a *Y. pestis*-specific probe was constructed. A 14-kb *Eco*RI chromosomal restriction fragment (E14) from *Y. pestis* 6/69, which contains the *irp2* gene (6), was cloned into a bacteriophage EMBL4 vector (Promega). The ligated DNA was packaged by using the Packagene system (Promega) and transfected into *E. coli* LE392. Recombinant *E. coli* colonies were screened by colony blotting with the *Y. enterocolitica* Ye8081 8-kb *Cla*I fragment (Cl8) carrying the *irp2* gene (6). The *hms* locus was obtained by insertion of a 10-kb *Bgl*II-*Sal*I fragment extracted from cosmid peH64 into the *Bam*HI-*Sal*I sites of plasmid PSU18. The recombinant plasmid was designated pSUhms. The other cosmid restriction fragments of interest were eluted from the agarose gel with the Geneclean kit (Bio 101, Inc.) and used directly as probes or cloned into the corresponding sites on the polylinker of the pBluescript II KS$^+$ plasmid (Stratagene, La Jolla, Calif.). To obtain a portion of the sequence inserted into the pigmentation segment of strain K169, the genomic DNA of this strain was digested with *Eco*RI and subjected to electrophoresis. Restriction fragments of approximately 3 kb were eluted from the gel and ligated into the corresponding site on the polylinker of the pBluescript II KS$^+$ plasmid (Stratagene). Following electroporation, transformed colonies of *E. coli* XL1Blue (Bio 101, Inc.) were selected on ampicillin (100 μg/ml)-containing agar plates. Recombinant colonies were screened by colony blotting with the EH2 probe from strain 6/69.

Cosmid library. Preparation of high-molecular-weight DNA from pYV-cured strain 6/69 was done mainly as described in reference 5. Briefly, chromosomal DNA was partially cleaved with *Sau*3A (Janssen Biochimica) and sized on a 10 to 40% sucrose gradient. DNA restriction fragments ranging from 35 to 50 kb were ligated into *Bam*HI-digested and alkaline phosphatase-treated cosmid vector pHC79 (BRL, Cergy Pontoise, France). Recombinant cosmids were packaged into Gigapack II Plus packaging extracts (Stratagene) and used to infect *E. coli* DH1. Approximately 800 recombinant colonies were spotted on nylon filters.

Establishment of the physical maps of the *pgm* locus and analysis of adjacent regions. To characterize the HPI of strain 6/69, recombinant *E. coli* colonies containing the cosmid library were hybridized with the E14 probe. Positive clones were isolated, and their cosmid DNA was extracted and digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Spe*I, and *Not*I. Restriction maps of the different cosmids were obtained after combinations of single and double digestions with these enzymes. The restriction fragments located at each extremity of the cosmids were used to screen the cosmid library in search of new recombinant clones. The same strategy was applied to the new clones for further chromosome walking. The DNA segments were considered to be inside the 102-kb region when they hybridized with the DNA of strain 6/69 only and outside it when they hybridized with the DNAs of both strains 6/69 and 6/69$^-$. The borders of the locus corresponded to DNA fragments which hybridized with both strains but with a different restriction pattern. The same criteria were used to define the limits of the deleted fragments in other strains of *Y. pestis*. To determine whether the chromosomal region flanking the left border of the *pgm* locus was the same in the different *Y. pestis* strains studied, two probes located in the vicinity of the border, of which one is internal (a 3-kb *Eco*RI-*Spe*I fragment designated ES3 [see Fig. 1A]) and the other is external (a 3.3-kb *Bam*HI segment designated B3.3 [see Fig. 1A]) to the iron acquisition segment, were hybridized with the *Spe*I-digested DNAs of the different strains. Recognition of the same 12-kb *Spe*I restriction fragment by both probes suggested that the ES3 and B3.3 fragments were adjacent, while recognition of different-size *Spe*I fragments indicated that B3.3 did not flank the iron acquisition segment. The same strategy was used for the right border of the pigmentation segment with the internal 3-kb *Eco*RI-*Cla*I (EC3) and the external 2.4-kb *Eco*RI-*Hin*dIII (EH2.4) probes (see Fig. 1A).

PCRs. Several of the sets of primers used in this study were described previously (5). In addition, new sets of primers were used. The sequences of the sense (SP) and antisense (ASP) primers, the sizes of the amplified fragments (S), and the annealing temperatures (A) for the PCR were as follows. (i) IS*285* (18): SP, 5′-TGGACGAAAAGAAAC-3′; ASP, 5′-AACAATGGGATACAG-3′; S, 488 bp; A, 50°C. (ii) *irp1* (18, 37): SP, 5′-AGAAACCGATGCTCACCC-3′; ASP, 5′-TCCTCTCCTGACGTAGCC-3′; S, 526 bp; A, 57°C. (iii) *psn* (16): SP, 5′-CTTTCCACCAACACCATCC-3′; ASP, 5′-AAACCGCCACTTCGCTTC-3′; S, 1,062 bp; A, 57°C. (iv) *ybtA* (14): SP, 5′-ACAGAGTCACCGCAAACG-3′; ASP, 5′-CAGATCAGCCAGCAGCAG-3′; S, 810 bp; A, 57°C. (v) *ybtE* (18, 37): SP, 5′-CCCTTACCCATTGCCGAAC-3′; ASP, 5′-TCCCCACCTCATCCAGCC-3′; S, 1,189 bp; A, 57°C. (vi) *ybtT* (37): SP, 5′-CCGCTCAGAAGCATTACAC AAC-3′; ASP, 5′-TCGCCGTCAATCACCACC-3′; S, 500 bp; A, 57°C. The template used for all PCRs was the genomic DNA of strain 6/69.

Pulsed-field gel electrophoresis. Genomic DNA was prepared in agarose plugs as previously described (4). Following digestion with *Spe*I or *Not*I, macrorestriction fragments were resolved by contour-clamped homogeneous electric field electrophoresis using a CHEF-DRIII apparatus (Bio-Rad Laboratories), an electric field of 6 V/cm, and an angle of 120°. Migration of the DNA fragments was determined in 0.5× Tris-borate-EDTA buffer and in 0.9% agarose gels maintained at 17°C. Pulse times were ramped from 1 to 10 s or from 1 to 18 s over 29 h for *Not*I- and *Spe*I-generated restriction fragments, respectively.

RESULTS

Physical and genetic maps of the 102-kb region of strain 6/69. The 102-kb region of *Y. pestis* 6/69 was characterized by chromosome walking on different overlapping cosmids (Fig. 1A) as described in Materials and Methods. The size of this region was estimated to be approximately 103 kb, similar to the 102 kb reported by Fetherston et al. (17) for strain KIM6$^+$. Its restriction map is shown on Fig. 1A. In addition to *irp2* and, probably, *irp1*, the other genes identified and positioned so far on the 102-kb region of strain KIM6$^+$ are the *psn* (16), *hms* (36), *ybtA* (14), *ybtE*, and *ybtT* (37) loci. Hybridization with a

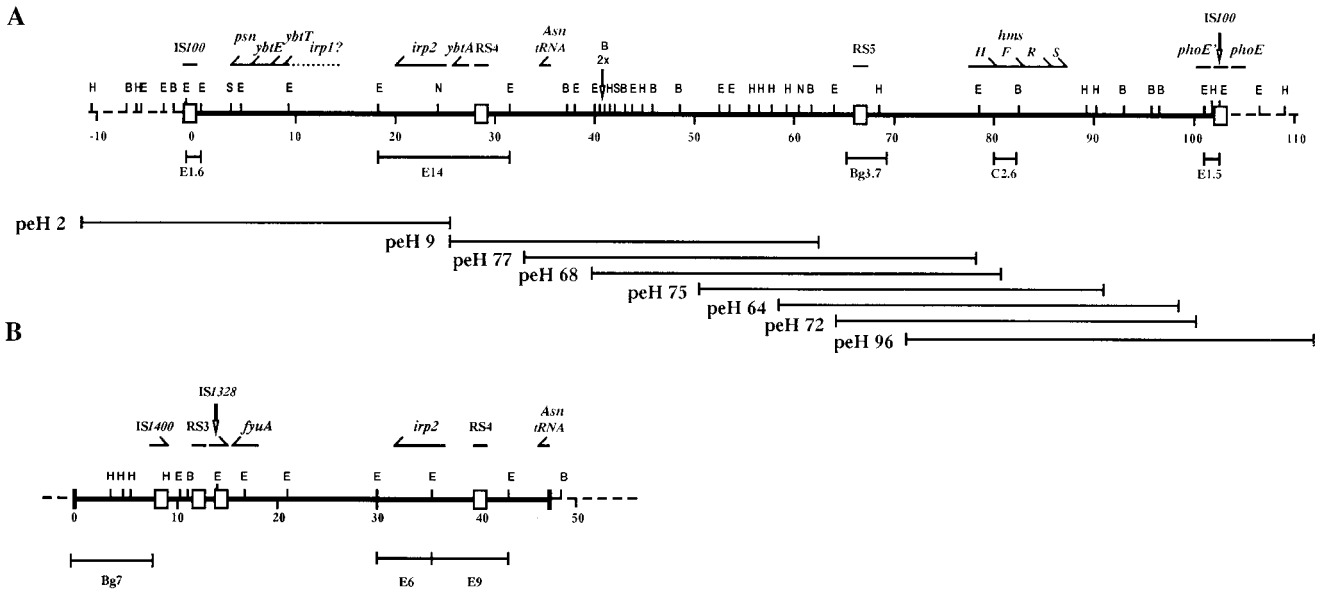

FIG. 1. Restriction maps of the 102-kb region of *Y. pestis* 6/69 (A) and the HPI of *Y. enterocolitica* Ye8081 (B). Plain thick lines, the 102-kb region; broken lines, regions outside the 102-kb region. Restriction sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Spe*I; N, *Not*I. Horizontal bars below the restriction maps represent the different probes cited in the text. The values below the map indicate the scale in kilobases. Horizontal arrows and bars above the restriction map correspond to the identified genes. peH, cosmid clones used to span the entire 102-kb region. RS, repeated sequences; 2×, two very close *Bam*HI restriction sites.

PCR-amplified portion of these genes indicated that they are also present on the 102-kb region of strain 6/69, on the same restriction fragments, and probably in the same positions as in strain KIM6$^+$ (Fig. 1A). It has also been shown that the 102-kb region of strain KIM6$^+$ is flanked by single copies of the IS*100* insertion sequence (IS) (39). This IS was found in multiple copies on the chromosome of strain 6/69 (Fig. 2). The fact that a PCR-amplified portion of this IS hybridized with an E1.6 fragment in cosmid peH2 and with an E1.5 fragment in cosmid peH96 but with no fragment in the other cosmids (Fig. 1A) indicated that the 102-kb region of strain 6/69 also carries a single IS*100* copy at each extremity but that no copy of the IS is present inside this locus.

A novel repeated sequence was identified on the pigmentation segment of strain 6/69, more precisely, on a 3.7-kb *Bgl*II restriction fragment (Bg3.7; Fig. 1A). It was designated repeated sequence 5 (RS.5) following RS.4 (5). This RS hybridized with more than 20 *Eco*RI fragments on the genome of strain 6/69 (Fig. 2), but a single copy was found on the 102-kb region of this strain. The specific *Eco*RI hybridization pattern of RS.5 clearly indicated that it is different from IS*100* (Fig. 2). Several other repeated sequences have been previously identified in *Yersinia* spp.: the IS*200*-like element which lies within the *inv* gene of *Y. pestis* (45), the IS*3*-like element identified downstream of the *ail* gene and specific for high-pathogenicity strains (34), the IS located downstream of the *yopE* gene of *Y. enterocolitica* (19), an approximately 100-bp intervening sequence that interrupts the 23S ribosomal DNA of pathogenic *Y. enterocolitica* (46), IS*285* of *Y. pestis* (18), and RS.3, RS.4, IS*1400* (5), and IS*1328* (41) identified on the HPI of *Y. enterocolitica*. None of the specific probes for these different repeated sequences hybridized with the cosmid clones encompassing the RS.5 gene, indicating that the novel repeated sequence is different from all of the repeated sequences previously identified in yersiniae. Study of its distribution among *Yersinia* spp. showed that it was present in multiple copies in the *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. mollaretii*, and *Y. bercovieri* strains tested and in low copy number (two or three)

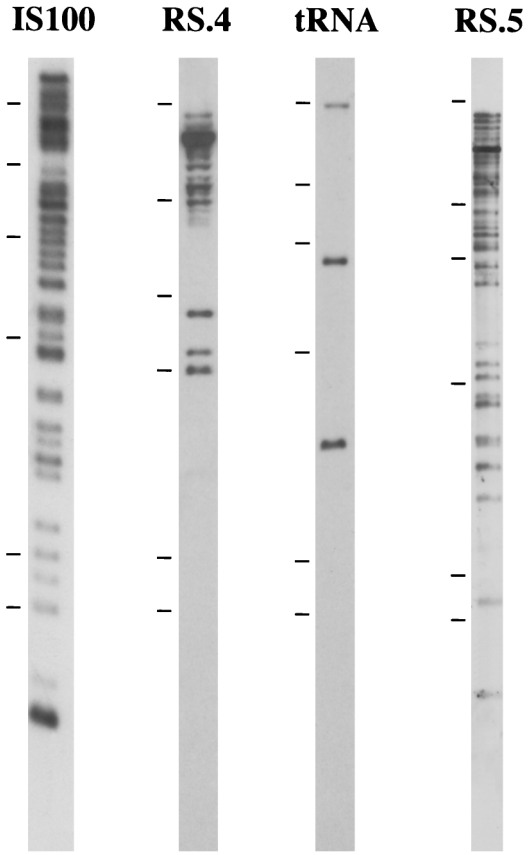

FIG. 2. Hybridization profiles of four different repeated sequences present in the 102-kb region of strain 6/69. The *Eco*RI-digested genomic DNA of strain 6/69 was hybridized with the E1.6 probe from *Y. pestis* 6/69 for IS*100* (Fig. 1A), the SCI1 probe from *Y. enterocolitica* Ye8081 for RS.4 (5), a 50-bp oligonucleotide probe from *Y. enterocolitica* Ye8081 for the *asn* tRNA (5), and the Bg3.7 probe from *Y. pestis* 6/69 for RS.5. The tick marks indicate the following molecular sizes, top to bottom: 23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kb.

in the *Y. intermedia*, *Y. kristensenii*, and *Y. frederiksenii* strains studied (data not shown). Therefore, neither the presence of RS.5 among *Yersinia* species nor its copy number is related to virulence.

Comparison of the HPI of *Y. enterocolitica* with the 102-kb region of *Y. pestis*. The 102-kb region of *Y. pestis* 6/69 has been represented in the sense opposite to that of the published partial map of the 102-kb region of strain KIM6$^+$ to compare its organization with that of the recently described HPI of *Y. enterocolitica* 1B (5). It has been shown previously that the HPI of *Y. enterocolitica* Ye8081 extends over 45 kb and is not flanked by repeated elements but harbors the *fyuA*/*psn*, *irp2*, and (probably) *irp1* loci (5). Comparison of the physical maps and gene locations of the HPI of *Y. enterocolitica* and of the 102-kb region of *Y. pestis* indicates good conservation of the 30-kb segment corresponding to the right part of the Ye8081 HPI (Fig. 1B). Confirmation of this observation was obtained by hybridizing the different probes that spanned the HPI of *Y. enterocolitica* Ye8081 (5) with the genomic DNAs of strains Ye8081 and 6/69. The probes located on the 30-kb right part of the HPI of strain Ye8081 recognized similar-size fragments in both species with the exception of the E6 and E9 probes (Fig. 1B), which both recognized an ≈14-kb *Y. pestis* fragment due to the absence of an *Eco*RI site in strain 6/69. Upstream of the *irp2* locus, a repeated element designated RS.4 was identified in all strains of *Y. enterocolitica* 1B previously analyzed (5). This repeated sequence was also present on the *Y. pestis* chromosome, in at least 16 copies (Fig. 2). Of these, only one was present on the iron acquisition segment of strain 6/69 and was located, as for *Y. enterocolitica*, upstream of the *irp2* gene (Fig. 1A and B). We also previously demonstrated that the HPI of *Y. enterocolitica*, like most of the pathogenicity islands described so far, is bordered on its right side by a copy of a tRNA gene (5). The use of a 50-bp oligonucleotide probe internal to the *asn* tRNA gene of *Y. enterocolitica* (5) indicated that this gene is present in three copies on the chromosome of strain 6/69 (Fig. 2) and that one copy is located at the same position as in *Y. enterocolitica* (Fig. 1A and B).

A divergence of the iron acquisition regions of the two species was observed downstream of the *fyuA*/*psn* locus, at a position which corresponded to a cluster of three repeated sequences (IS*1400*, RS.3, and IS*1328*) in *Y. enterocolitica* (Fig. 1). IS*1400* and RS.3 have already been shown to be absent from the chromosome of strain 6/69 (5). IS*1328* was initially found in *Y. pestis* (5), but when a probe internal to the IS was used, no signal was detected, suggesting that the hybridizing DNA lay outside the IS. The divergence of the two regions concerned not only the repeated sequence but also the nonrepeated adjacent DNA, since the *Y. enterocolitica* Bg7 probe (Fig. 1B) recognized a very faint band of a different size in *Y. pestis* 6/69. This interspecies difference was not surprising, since within the species *Y. enterocolitica* itself, this left portion of the HPI was the less conserved (5). Therefore, our results demonstrate that a fragment of 30 kb of the HPI of *Y. enterocolitica* is very well conserved in *Y. pestis* while the remaining 15-kb left portion of the HPI diverged in the two species.

When the reverse experiments were performed, i.e., when the *Y. pestis* probes were used to hybridize with the *Y. enterocolitica* chromosome, the probes covering the pigmentation segment of strain 6/69 hybridized faintly or not at all with the DNA of *Y. enterocolitica*, indicating that, as previously observed by other researchers with another strain (17), the pigmentation segment of *Y. pestis* is absent or degenerate in *Y. enterocolitica*.

Analysis of the mechanisms responsible for the existence of nonpigmented *Y. pestis* mutants harboring the *irp2* locus. In a previous study (27), we found that 7 of the 43 strains of *Y. pestis* analyzed were nonpigmented but harbored the *irp2* locus (Pgm$^-$ Irp2$^+$). In the present study, the availability of cosmid clones spanning the *hms* locus allowed us to further analyze these mutants. In addition, two new Pgm$^-$ Irp2$^+$ derivatives obtained in vitro from Pgm$^+$ Irp2$^+$ parental strains (Kenya 169 and Saigon 55-1239) were included in the study (Table 1). Two categories of strains were distinguished. The first category included the two strains from Turkey and the three strains from Kenya whose DNA did not hybridize with a portion of the *hms* locus (C2.6 probe; Fig. 1A), indicating deletion of at least a part of this locus. The second category was composed of the strains from Germany, Vietnam, and Zaire which did harbor the *hms* locus although they were nonpigmented. To characterize the mechanisms responsible for the Pgm$^-$ Irp2$^+$ phenotype, we mapped the 102-kb regions of all nine isolates.

Characterization of the two Pgm$^-$ Irp2$^+$ strains from Turkey. Hybridization experiments with probes covering the 102-kb region of strain 6/69 (Fig. 1A) revealed that the same deletion event occurred in the two strains from Turkey and involved not only the *hms* locus but at least 70 kb of DNA (Fig. 3B).

The left border of the deletion was identified on a 1.4-kb *Cla*I segment (C1.4; Fig. 3) which carries the *asn* tRNA gene. Hybridization with a 50-bp oligonucleotide probe internal to the *asn* tRNA gene demonstrated that at least a portion of this locus was removed during the deletion process. On the right side, the deletion extended up to the right border of the pigmentation segment (Fig. 3). Whether the right IS*100* copy was also removed was difficult to determine because of the high number of hybridizing fragments generated by the DNA segment carrying the IS*100* sequence and the polymorphism of the patterns observed between strains of different origins. To indirectly answer this question, we looked for the occurrence of spontaneous Pgm$^-$ Irp2$^-$ derivatives of the Pgm$^-$ Irp2$^+$ Turkish strains. If a copy of IS*100* remained at the right of the truncated 102-kb region, homologous recombination with the copy present at the left end of the iron acquisition segment should occur, leading to Pgm$^-$ Irp2$^-$ derivatives. No such mutants could be obtained with strains T10/1 and T10/3, suggesting that the deletion internal to the 102-kb region also removed the right IS*100* element and thus prevented secondary deletion of the remnant 33-kb left portion of this locus. The absence of Pgm$^+$ Irp2$^+$ parental strains from Turkey did not allow us to determine the precise size of the deleted fragment and its mechanism of excision.

The 33-kb left part of the 102-kb region that was not deleted in the two Turkish strains had an *Eco*RI restriction map almost identical to that of strain 6/69. The only exception was found in the 8.8-kb *Eco*RI fragment located on the iron acquisition segment which was slightly larger in strains T10/1 and T10/3, suggesting an insertion of ≈500 bp (Fig. 3B). An IS*100* element was also found at the left border of the iron acquisition segment of the two strains from Turkey, and their chromosomal regions flanking this border were similar to that of strain 6/69.

Altogether, our results indicate that the nonpigmented phenotype observed in the two Pgm$^-$ Irp2$^+$ Turkish strains is due to a large (≥70-kb) chromosomal deletion involving the *asn* tRNA, the entire pigmentation segment, and the right IS*100* copy and that the remaining 33-kb left part of the 102-kb region displays a high degree of conservation with respect to the iron acquisition segment of strain 6/69.

Characterization of the three Pgm$^-$ Irp2$^+$ strains from Kenya. In Pgm$^-$ Irp2$^+$ strains K129, K164, and K169.1 from Kenya, the sizes of the deletions involving the *hms* locus were

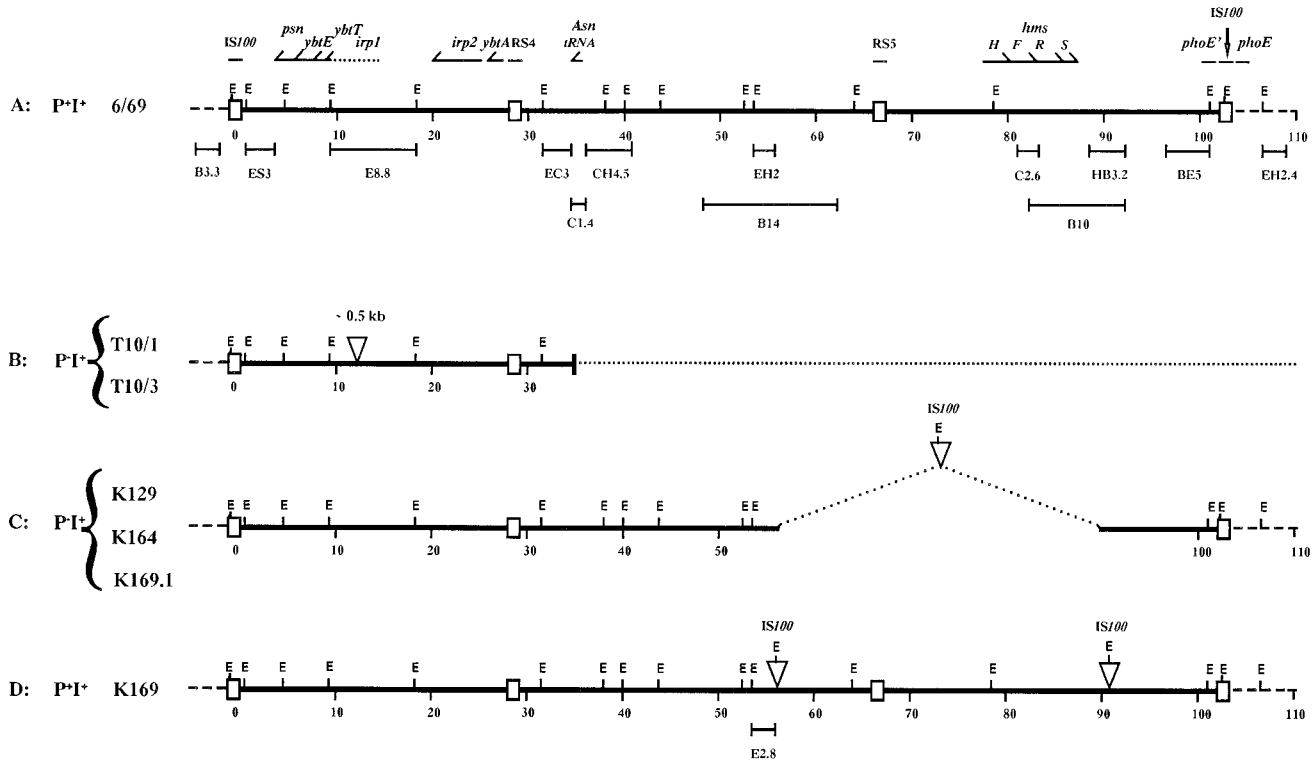

FIG. 3. Comparison of the 102-kb region *Eco*RI restriction map of strain 6/69 (A) with those of strains from Turkey (B) and Kenya (C and D). Plain thick lines, the 102-kb region; broken lines, regions outside the 102-kb region; dotted lines, deleted regions; E, *Eco*RI restriction sites; triangles, DNA sequences that were absent from the 6/69 102-kb region. Horizontal bars below the restriction maps represent the different probes cited in the text. The values below the map indicate the scale in kilobases. Horizontal arrows and bars above the restriction map correspond to the identified genes. RS, repeated sequences; P^+I^+, Pgm^+ $Irp2^+$; P^-I^+, Pgm^- $Irp2^+$.

the same: approximately 38 kb (Fig. 3C). By walking outward from the *hms* locus, the left limit of the deletion was located on a fragment corresponding to the 2-kb *Eco*RI-*Hin*dIII fragment (EH2) of strain 6/69, and the right limit was on a 3.2-kb *Hin*dIII-*Bam*HI fragment (HB3.2; Fig. 3A).

The junction fragment generated after excision of the 38-kb intervening DNA contained an additional *Eco*RI site that was absent in strain 6/69. Analysis of Pgm^+ $Irp2^+$ parental strain K169 from which Pgm^- $Irp2^+$ strain K169.1 was derived revealed that, by comparison with the 6/69 restriction map, two additional 2-kb sequences each carrying an *Eco*RI site were present on the pigmentation segment of this strain. Their positions corresponded to the limits of the 38-kb unstable fragment in the three Pgm^- $Irp2^+$ Kenya strains (Fig. 3D). To determine the nature of these additional sequences, a 2.8-kb *Eco*RI fragment from strain K169 (E2.8; Fig. 3D) encompassing a portion of this sequence was cloned into pBluescript. The cloned insert hybridized with multiple *Eco*RI fragments in strains K169 and 6/69 and displayed a hybridization pattern identical to that generated by the IS*100* element. This indicates that a copy of IS*100* flanks the left border, and most probably the right border, of the 38-kb unstable fragment in the Kenya strains (Fig. 3D).

Outside the 38-kb unstable fragment, the *Eco*RI restriction maps of the 54-kb DNA segment located on its left side and the 11-kb DNA segment located on its right side were identical to those of the corresponding regions of strain 6/69 (Fig. 3C), demonstrating good conservation of the remaining portion of the 102-kb region in the strains from Kenya. Furthermore, the restriction map of the 38-kb unstable segment of parental strain K169 was also identical to that of strain 6/69 (Fig. 3D). The presence of intact IS*100* sequences flanking the 102-kb region was indirectly demonstrated by the fact that Pgm^- $Irp2^-$ derivatives of the Pgm^- $Irp2^+$ strains could be obtained. The chromosomal region flanking the right border of the pigmentation segment was also conserved in the Kenya and 6/69 strains, while the region to the left of the iron acquisition segment differed between these two groups of strains (data not shown).

Our data demonstrate that the nonpigmented phenotype of strains K129, K164, and K169.1 from Kenya is due to a deletion of 38 kb of DNA, most probably mediated by homologous recombination between two additional IS*100* copies flanking the unstable fragment in the Kenyan strains.

Characterization of the four Pgm^- $Irp2^+$ strains retaining the *hms* locus. As mentioned above, the four remaining Pgm^- $Irp2^+$ strains, H19, CBL, S55-1239.1, and S55-797, hybridized with the C2.6 probe, indicating that they did not undergo deletion of this region. Further analysis of their 102-kb regions and comparison with that of strain 6/69 were undertaken.

(i) Analysis of strain Hamburg 19. The 102-kb region *Eco*RI restriction maps and chromosomal flanking regions of strains H19 and 6/69 were almost identical. The only minor differences corresponded to the presence of two DNA insertions of approximately 1.5 kb, both carrying one additional *Eco*RI and *Bam*HI restriction site (Fig. 4B). The presence of the same two restriction sites in the two additional sequences and their similar sizes suggest that they correspond to the insertion of the same element at two different positions on the *pgm* locus of strain H19.

Since these two DNA insertions were outside the *hms* locus, they could not account for the nonpigmented phenotype of this

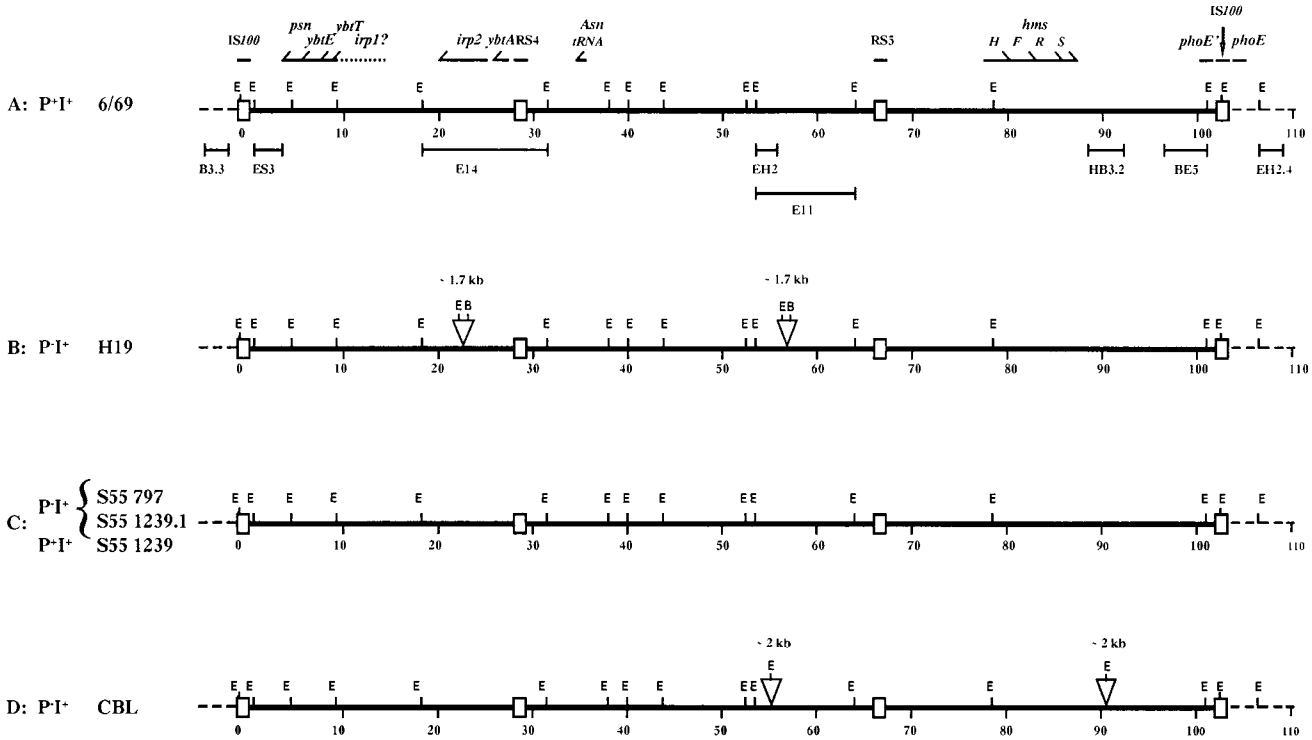

FIG. 4. Comparison of the 102-kb region *Eco*RI restriction map of strain 6/69 (A) with those of strains from Hamburg (B), Ho Chi Minh City (C), and Zaire (D). Plain thick lines, the 102-kb region; broken lines, regions outside the 102-kb region; E, *Eco*RI restriction sites; triangles, DNA sequences that were absent from the 6/69 102-kb region. Horizontal bars below the restriction maps represent the different probes cited in the text and used to analyze the strains. The values below the map indicate the scale in kilobases. Horizontal arrows and bars above the restriction map correspond to the identified genes. RS, repeated sequences.

strain. The absence of pigmentation could be due either to point mutations in the *hms* locus or to rearrangements in a gene (structural or regulatory) contributing to the pigmentation phenotype but different from the *hms* locus. To distinguish between these two possibilities, a complementation study with the *hms* genes was performed. Perry et al. (36, 38) previously demonstrated that a 9.1-kb cloned DNA fragment that carries the *hms* genes could restore the pigmentation phenotype of a strain following deletion of the entire 102-kb region. We cloned a 10-kb *Bgl*II-*Sal*I fragment that encompasses the 9.1-kb fragment and the four *hms* genes (31). Electroporation of recombinant plasmid pSUhms into strain 6/69$^-$ (in which the entire 102-kb region is deleted), conferred a red color on the colonies grown on Congo red-agar plates, confirming that pSUhms can complement the loss of the *hms* locus. However, the coloration of the 6/69$^-$(pSUhms) clones was not as intense as that of wild-type strain 6/69, suggesting that other genes in the 102-kb region contribute to the pigmented phenotype. Introduction of pSUhms into strain H19 produced red transformants similar in color to strain 6/69. Therefore, loss of pigmentation in strain Hamburg 19 results from a mutation in the *hms* locus, the same mechanism previously reported by Fetherston and Perry for strain M23 (16).

(ii) Analysis of the two Pgm$^-$ Irp2$^+$ strains from Ho Chi Minh City. The 102-kb region *Eco*RI restriction maps and chromosomal flanking regions of Pgm$^-$ Irp2$^+$ strains S55-797 and S55-1239.1 (Fig. 4C) and Pgm$^+$ Irp2$^+$ parental strain S55-1239 were identical to those of strain 6/69.

Since no chromosomal rearrangements were detected to account for the nonpigmented phenotype of the two Saigon strains, complementation experiments with pSUhms were undertaken. Strain S55-1239.1 yielded red colonies on Congo red-agar plates upon complementation with pSUhms, indicating that, as for strain H19, loss of pigmentation was due to a mutation in the *hms* locus. Strain S55-797 behaved differently, since even before introduction of the recombinant plasmid, the colonies were not plain white but very light pink. Completely white Pgm$^-$ Irp2$^-$ colonies in which the entire 102-kb region had been deleted could be derived from this strain. However, introduction of pSUhms in strain S55-797 did not restore pigmentation of the recipients, which remained very light pink. Altogether, these results suggest that an alteration in a gene present in the 102-kb region and contributing to the pigmentation phenotype but different from the *hmsHFRS* genes is responsible for the Pgm$^-$ Irp2$^+$ phenotype of strain S55-797. To test this hypothesis, cosmid peH64, which encompasses approximately 40 kb of the 102-kb region of strain 6/69 (Fig. 1A), was introduced into strain S55-797. The recombinant colonies acquired a red coloration, indicating that a gene present on the pigmentation segment, more precisely, on the 40-kb region covered by peH64, contributes to the pigmented phenotype of *Y. pestis* and is mutated in strain S55-797. This gene was not further characterized.

(iii) Analysis of the Pgm$^-$ Irp2$^+$ strains from Zaire. The *Eco*RI restriction map of the 102-kb region of strain CBL closely resembled that of strain 6/69 (Fig. 4D). The only differences were the insertion of two 2-kb DNA fragments containing an *Eco*RI restriction site. These inserted sequences were located outside the *hms* locus and therefore could not explain the nonpigmented phenotype. Attempts to complement strain CBL with pSUhms were unsuccessful because the strain did not grow well enough in vitro for preparation of electrocompetent cells.

Strikingly, the two 2-kb additional sequences of strain CBL

were inserted at the same positions on the pigmentation segment as the internal IS*100* sequences of strain K169 (Fig. 3D and 4D). The facts that the two inserted sequences of strain CBL carried an *Eco*RI site, were of the same size, and were located at the same position on the pigmentation segment as the IS*100* copies of strain K129 strongly suggest that they also are IS*100* copies. Indirect confirmation of this hypothesis was obtained from the fact that, as with strain K169, spontaneous deletion of the 38-kb DNA fragment located between the two inserted sequences was observed in strain CBL. The similarity between strains CBL and K169 extended outside the 102-kb region to the chromosomal flanking regions.

Therefore, the 102-kb region of the *Y. pestis* strain from Zaire is very similar to that of the strains from Kenya in its physical map and is also subject to spontaneous deletion of a 38-kb DNA fragment located between two inserted sequences that are probably additional IS*100* sequences.

DISCUSSION

In this study, the 102-kb DNA segment constituting the *pgm* locus of *Y. pestis* 6/69 (a virulent strain from Madagascar) has been identified and its restriction map has been established. This locus has the same size, restriction map, gene location, and flanking IS*100* copies as those previously established for strain KIM6$^+$ (16, 17), indicating a high degree of conservation among strains of different origins and biotypes. This observation was reinforced by the fact that the *Eco*RI physical map of the entire 102-kb region was almost the same in all *Y. pestis* strains of various geographical origins (Hamburg, Ho Chi Minh City, Zaire, Madagascar, and Kenya) tested and over the remnant of the 102-kb region still present in the strains from Turkey. The minor differences observed concerned the presence in some strains of one or two additional, small DNA segments. However, these differences were limited to one or two positions in the 102-kb region and resulted in a difference in size not exceeding a total of 4 kb.

The 102-kb region of *Y. pestis* is composed of two physically and functionally distinct portions: a ca. 35-kb segment (left part in Fig. 1A), which is involved in siderophore-mediated iron acquisition, and a ca. 68-kb segment (the right part), which carries genes conferring the pigmentation phenotype. Although this arrangement of pathogenicity-associated genes flanked by ISs superficially resembles a pathogenicity island (24), there is little evidence that both parts have ever formed a horizontally transmitted unit. The fact that the right IS*100* flanking copy disrupted the *phoE* gene in KIM strains (16) indicates that the entire 102-kb locus was not transposed en bloc into this region. Insertion of IS*100* into this region of the *Y. pestis* chromosome seems to be a frequent phenomenon. Sequence data suggest that the two parts of the 102-kb region have different origins: the background GC content of yersiniae is 46 to 50%, and the 7.6-kb *hms* operon involved in pigmentation has a GC content of 46.9% (36, 38), while the overall GC contents of the fully sequenced *Y. pestis* iron acquisition genes (*psn*, *ybtE*, *ybtT*, and *ybtA*) (1, 14, 15) and of the *Y. enterocolitica irp2* sequence (20) are 55.8 and 59.2%, respectively. Another piece of evidence that there are two distinct units comes from the fact that most of the iron acquisition segment is well conserved in *Y. enterocolitica* and *Y. pestis*, while the pigmentation segment is either absent or highly degenerated in the former species. Furthermore, most of the *Yersinia* iron acquisition segment is also present as a unit in various pathogenic *E. coli* strains (43).

The iron acquisition segment of *Y. pestis* is downstream of a tRNA locus, carries genes necessary for the expression of a high-pathogenicity phenotype, has a GC content different from that of the remainder of the chromosome, is homologous to the HPI of *Y. enterocolitica*, and is found in other enterobacterial species. Therefore, this 35-kb left portion of the 102-kb region of *Y. pestis* can be considered an HPI per se.

We found no evidence for mobility of the HPI independently of the pigmentation segment. Similarly, Fetherston and Perry (16) could not identify any *Y. pestis* strain with the HPI portion of the 102-kb region alone deleted. These results are consistent with those obtained with *Y. enterocolitica*, since we were able to identify neither spontaneous HPI deletion mutants in our culture collection nor deleted colonies following repeated subculture of strain Ye8081 (11). Although the HPI of *Y. pestis* is not deleted independently of the pigmentation segment, the two regions do not display the same degree of in vitro stability. Indeed, several Pgm$^-$ Irp2$^+$ *Y. pestis* strains were previously identified (27). This study reveals that different mechanisms could cause the nonpigmented phenotype: (i) deletion of an internal 38-kb DNA segment within the pigmentation segment mediated by homologous recombination between two additional internal copies of IS*100*, (ii) deletion of over 70 kb of DNA involving the right portion of the HPI and probably the adjacent chromosomal region, (iii) mutation in the *hms* locus, and (iv) alteration of an unidentified gene located in the pigmentation segment and contributing to the pigmented phenotype but different from the *hms* locus.

Our demonstration of these different types of deletion or mutation within the 102-kb region conflicts with the high degree of conservation of this region overall among strains that differ in origin. However, it should be emphasized that the instability of the 102-kb region and its complete loss on subculture (11) are in vitro phenomena. Actually, all fresh isolates previously studied harbored the *irp2* gene (12), indicating that the 102-kb region is maintained under natural conditions, probably because it encodes functions essential for in vivo survival (iron acquisition system) and for transmission to new hosts via flea vectors (hemin storage system [26, 30]). The observation that the geographical origin of the *Y. pestis* strains correlates with the type of event responsible for the nonpigmented phenotype suggests that specific genomic rearrangements which secondarily led to the nonpigmented phenotype in vitro occurred in each ecosystem. Adaptive genomic modifications generated during the life cycle of a bacterium in the natural environment, followed by clonal expansion, would thus predispose strains from a given geographical origin to certain specific types of rearrangements. Such a clonal expansion of new variants of *Y. pestis* has recently been demonstrated in the plague foci of Madagascar (22).

Devignat postulated that *Y. pestis* strains of biotypes Antiqua, Medievalis, and Orientalis were responsible for the first, second, and third plague pandemics, respectively (13). Recent results obtained with molecular typing techniques such as pulsed-field gel electrophoresis or ribotyping of strains from different regions have supported this conjecture (21, 32, 40). The four strains of biotype Antiqua examined in this study (strains K129, K164, and K169 from Kenya and strain CBL from Zaire) had identical characteristic features for their 102-kb regions: (i) similar chromosomal flanking regions, (ii) two IS*100* insertions at the same position on the pigmentation segment, and (iii) instability of the region located between these two inserted sequences. These features were not found in the 102-kb region of the biotype Orientalis strains studied, reinforcing Devignat's hypothesis about the clonality of the strains of each biotype. However, these interbiotype differences within the 102-kb region are minor. If we accept the association of different biotypes with different pandemics, then the 102-kb region of *Y. pestis* is a stable feature of strains

associated with outbreaks from the 5th century up to the present day.

In conclusion, this study demonstrates that the 102-kb region of *Y. pestis* is composed of two clearly distinct parts, an HPI and a pigmentation segment, but these two components are well conserved and stably linked in different strains, probably because they are essential for bacterial survival under natural conditions. Both the originally observed deletion of the entire 102-kb region (17) and the additional mutations we have defined leading to the nonpigmented phenotype are probably in vitro phenomena and are consequences of the presence of numerous ISs in the *Y. pestis* chromosome. Nonetheless, we believe that these different mutations reflect an underlying specific genomic adaptation of these strains to their local environment, followed by clonal expansion in the ecosystem.

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