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Characterization of a Large Chromosomal "High-Pathogenicity Island" in Biotype 1B *Yersinia enterocolitica*

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Pathogenic *Yersinia* spp. can be subdivided into highly pathogenic (high-pathogenicity) and low-pathogenicity strains. Several genes specific for the high-pathogenicity strains are clustered on a chromosomal fragment designated a "high-pathogenicity island" (HPI). In the present work, the HPI of biotype 1B strain Ye 8081 of *Y. enterocolitica* was characterized. We demonstrate important differences from the HPI of *Y. pestis*. The HPI of *Y. enterocolitica* is smaller (45 kb) and is not flanked by insertion sequences. A copy of the gene coding for the tRNA-Asn is present at one extremity of the HPI and may, as in uropathogenic *Escherichia coli*, participate in the excision of the island. In addition to the genes encoding the yersiniabactin-pesticin receptor and the high-molecular-weight protein 2, four repeated sequences are present on the HPI of *Y. enterocolitica*. At least two of them are insertion elements: previously described IS*1328* and newly characterized IS*1400*. Comparison of the HPI of strain Ye 8081 with that of other *Y. enterocolitica* strains of biotype 1B indicates that most of the island is conserved, apart from 15 kb at the left-hand end which is variable, especially in the region where three repeated sequences are clustered.

Yersinia spp. are gram-negative bacteria belonging to the *Enterobacteriaceae* family. The genus is composed of several species which can be divided into three main pathogenicity groups. Nonpathogenic strains are found in the environment and sometimes as transient intestinal saprophytes in humans, but they do not cause clinical infections. These include *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. mollaretii*, *Y. bercovieri*, *Y. aldovae*, *Y. rohdei*, *Y. ruckeri*, and biotype 1A of *Y. enterocolitica*. Strains with low levels of pathogenicity (low-pathogenicity strains) are widespread in cold and temperate countries. They are responsible for mild enteric infections in humans and are not lethal for mice at low doses. They correspond to *Y. enterocolitica* strains of biotypes 2 to 5. Finally, high-pathogenicity strains are lethal for mice at low doses and induce severe, sometimes fatal diseases in humans. This group comprises *Y. pestis* (the plague bacillus), *Y. pseudotuberculosis*, and biotype 1B strains of *Y. enterocolitica.*

Several genes have been cloned and shown to be present only in high-pathogenicity strains: (i) the *hms* locus (30) encoding the capacity to store hemin at the cell surface and thus giving a pigmented phenotype (Pgm) to *Y. pestis* colonies grown on hemin or Congo red agar plates (44), (ii) the *fyuA-psn* gene (11, 36) coding for a protein (FyuA) which acts as the receptor for two distinct molecules, the siderophore yersiniabactin and the bacteriocin pesticin (19), and (iii) the *irp2* gene (8) encoding the iron-regulated high-molecular-weight protein 2, which participates in the nonribosomal synthesis of an unidentified small peptide which may be a siderophore (17). Strikingly, in *Y. pestis*, these three loci are clustered on a 102-kb chromosomal fragment, the *pgm* locus (12, 13), which is flanked by single copies of the IS*100* insertion element (12, 31).

Deletion of the *pgm* locus is a frequent phenomenon in *Y. pestis* (5, 10) and probably results from homologous recombination between the flanking copies of IS*100*. Loss of the *pgm* locus is characterized by a nonpigmented phenotype and is accompanied by a marked reduction in the pathogenicity of the strain upon subcutaneous injection with a 1,000-fold increase in 50% lethal dose (LD_{50}) for mice (20), but a deletion mutant strain is still able to multiply in vivo, and its LD_{50} is equivalent to that of a wild-type strain by intravenous injection (7). Therefore, deletion of the *pgm* locus does not abolish the capacity to cause disease but prevents the most fulminant expression of bacterial pathogenicity. This locus, whose presence is associated with the high-pathogenicity phenotype, can thus be called a "high-pathogenicity island" (HPI).

The high-pathogenicity strains of *Y. enterocolitica* 1B are naturally nonpigmented and do not possess an *hms* locus (13). However, they do harbor functional *irp2* and *fyuA* genes, and it has recently been shown that the two loci are separated by only ≈12 kb on the chromosome of *Y. enterocolitica* WA (35). Since these two genes are specific for high-pathogenicity strains and are physically linked, they can be regarded as components of an as yet uncharacterized HPI in *Y. enterocolitica*. The HPI of this species appeared much more stable than that of *Y. pestis*, since we were able to identify neither spontaneous biotype 1B *irp2* deletion mutant strains in our culture collection nor *irp2* deleted colonies following repeated subculture of high-pathogenicity strain Ye 8081 (10). However, Rakin and Heesemann recently reported the isolation of colonies from strain WA in which either the *fyuA* gene alone or both the *fyuA* and *irp2* loci had spontaneously deleted (35). Therefore, excision of the HPI of *Y. enterocolitica* 1B may also occur in some strains, although at a much lower frequency than in *Y. pestis.*

In the present study, we report the size and physical map of the HPI of Ye 8081, a *Y. enterocolitica* 1B strain. The boundaries of the island were defined, and several different repeated sequences located on the HPI and elsewhere on the chromosome were identified and characterized. Finally, the HPIs of various high-pathogenicity strains of *Y. enterocolitica* were

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TABLE 1. Characteristics of the *Yersinia* strains and distribution of the RS

Strain	Biotype and/or serotype[a]	Level of pathogenicity[b]	Presence of RS[c]				
			tRNA	IS*1400*	IS*1328*	RS.3	RS.4
Y. pestis 6/69M	Orientalis	H	+	−	+	−	+
Y. pseudotuberculosis							
IP 32954	I	H	+	+	−	±	+
IP 32938	II	H	+	+	−	±	+
Y. enterocolitica							
Ye 8081	1B/O:8	H	+	+	+	+	+
WA	1B/O:8	H	+	+	+	+	+
IP 1105	1B/O:8	H	+	+	+	+	+
IP 1110	1B/O:21	H	+	+	+	+	+
IP 24762	1B/O:21	H	+	+	+	+	+
IP 845	1B/O:20	H	+	+	+	+	+
IP 24766	1B/O:20	H	+	+	+	+	+
IP 24764	1B/O:13-20	H	+	+	+	+	+
IP 24763	1B/O:8-13	H	+	+	+	+	+
IP 24761	1B/O:4,32-4,33	H	+	+	+	+	+
IP 864	4/O:3	L	+	+	+	−	+
IP 21693	4/O:3	L	+	+	+	−	+
IP 383	2/O:9	L	+	+	+	−	+
IP 21349	2/O:9	L	+	+	+	−	+
IP 885	2/O:5,27	L	+	−	+	−	+
IP 24636	2/O:5,27	L	+	−	+	−	+
IP 20352	1A/O:5	N	+	−	+	−	+
IP 21711	1A/O:6,30-6,31	N	+	−	+	±	+
IP 21440	1A/O:7,8,19	N	+	−	+	−	+
IP 18970	1A/O:13	N	+	−	+	−	+
Y. intermedia							
IP 21356	4/O:8-8,19	N	+	−	+	+	+
IP 21533	1/O:4,33-16-16,29	N	+	−	+	−	+
Y. kristensenii IP 21577	O:12,25-12,26	N	+	−	+	+	+
Y. mollaretii IP 21081	O:49,51	N	+	−	−	−	+
Y. bercovieri IP 21531	O:36	N	+	−	−	−	+
Y. frederiksenii P 21689	O:16-16,29	N	+	−	+	+	+

[a] When both biotype and serotype appear, they are divided by a slash.
[b] H, high pathogenicity; L, low pathogenicity; N, nonpathogenic strain.
[c] The DNA of each strain was digested with *Eco*RI and hybridized with the different probes described in the legend to Fig. 4. +, contains one or several copies of the RS; −, contains no copy of the RS; ±, presence of faint hybridizing bands.

compared with that of strain Ye 8081 to evaluate the degree of conservation of the island within this species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The characteristics of the *Yersinia* strains used in this study are listed in Table 1. The three *Escherichia coli* strains used in this study were DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*), Y1090 (Δ*lac* U169 *proA*$^+$ Δ*lon araD139 strA supF trpC22*::Tn*10* [PMC9 Amp^r Tet^r]), 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.

DNA techniques. Isolation and digestion of genomic DNA were performed as previously described (8). Plasmid extractions were carried out according to the method of Birnboim and Doly (3). Double-strand DNA labeling was performed either radioactively with [^{32}P]dATP (Amersham, Les Ulis, France) or nonradioactively by the enhanced chemiluminescence reaction technique (ECL; Amersham). Single-strand oligonucleotides were labeled with the 3′-oligonucleotide labeling system (ECL; Amersham).

Cloning methodologies. The 6-kb *Eco*RI chromosomal restriction fragment (E6) from strain Ye 8081 which partially overlaps the *irp2* gene (6) was cloned into bacteriophage lambda gt10 vector (Promega). The ligated DNA was packaged by using the Packagene system (Promega) and transfected into *E. coli* Y1090. Recombinant *E. coli* colonies were screened by colony blotting with the 8-kb *Cla*I fragment (Cl8) carrying the *irp2* gene (6). The different cosmid restriction fragments of interest were eluted from the agarose gel with the Geneclean Kit (Bio 101 Inc., La Jolla, Calif.) and cloned into the corresponding sites on the polylinker of the pBluescript II KS$^+$ plasmid (Stratagene, La Jolla, Calif.). 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 homologous insert.

Cosmid library. Preparation of high-molecular-weight DNA from pYV-cured strain Ye 8081 was prepared mainly as described in reference 24. After extensive dialysis against 10 mM Tris (pH 7.5)–1 mM EDTA (pH 8), 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 the *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 750 recombinant colonies were spotted onto nylon filters and hybridized with the E6 and Cl8 probes (Fig. 1A). Colonies that hybridized with both probes were isolated, and their cosmid DNA was extracted.

Probe preparation. Restriction maps of two cosmids, pHC15 and pHC25, were obtained after combinations of single and double digestions with the *Eco*RI,

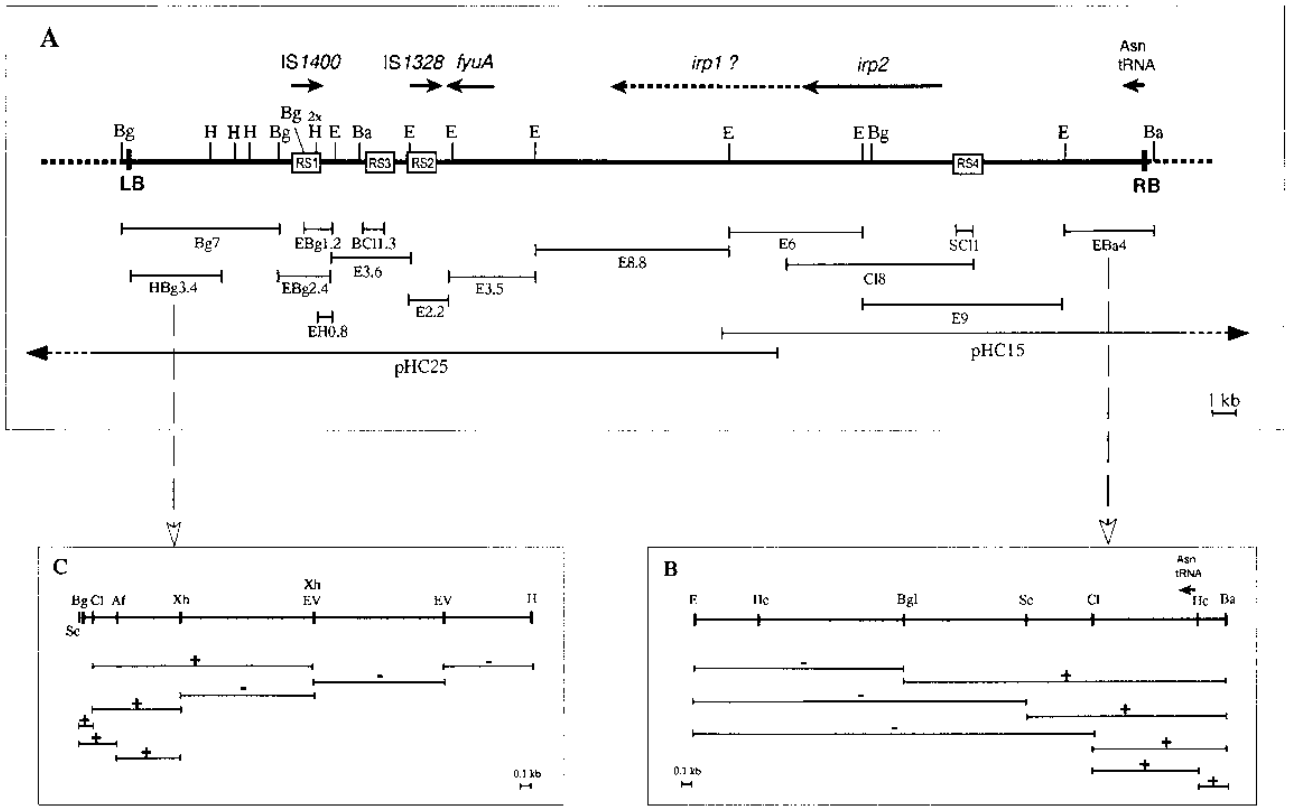

FIG. 1. Physical map (A) and definition of the right (B) and left (C) boundaries of the HPI of strain Ye 8081 of *Y. enterocolitica*. Horizontal bars below the restriction maps represent different inserts that have been used as probes to map the region. Arrows above the restriction map indicate the locations of the identified genes. Abbreviations and symbols: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; Bg, *Bgl*II; BgI, *Bgl*I; Sc, *Sac*II; Cl, *Cla*I; Ba, *Bam*HI; Xh, *Xho*I; Cl, *Cla*I; Af, *Afl*II; pHC25 and pHC15, cosmid clones; RB and LB, right and left boundaries; 2x, two very close *Hin*dIII restriction sites; +, probes that hybridized with the DNA of both the high-pathogenicity (Ye 8081) and low-pathogenicity (IP 383 and IP 864) strains; −, probes that hybridized only with the DNA of the high-pathogenicity strain.

*Bam*HI, *Bgl*II, and *Hin*dIII restriction enzymes. To obtain probes overlapping the entire HPI (Fig. 1A), various restriction fragments were excised from agarose gels with the Geneclean Kit (Bio 101 Inc.). For the PCR-amplified DNA, the products of the reactions were either loaded onto a gel and purified by electroelution or, when a single band was observed, directly used for labeling by the enhanced chemiluminescence reaction method (ECL; Amersham).

Sequencing. To sequence the entire RS.1 and the *asn-tRNA* gene, overlapping inserts were obtained by different strategies. First, several small subclones were directly ligated into the polylinker of pBluescript II KS$^+$ plasmid (Stratagene). Second, large inserts were treated with exonuclease III (Erase-a-base system; Promega Biotech) to generate a set of smaller fragments (2) which were inserted into the polylinker of pBluescript II KS$^+$ plasmid. Third, oligonucleotides were synthesized for nonoverlapping regions with a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The nucleotide sequences of the two DNA strands were determined by the dideoxy-chain termination method (2), with a modified T7 DNA polymerase. The nucleotide and amino acid sequences were analyzed with DNA Strider software (25), and the sequence library searching was carried out with the BLAST (1) and FASTA (29) programs.

PCRs. The different sets of primers used for PCR amplification were synthesized by Eurogentec (Seraing, Belgium). The sequences of the sense (SP) and antisense (ASP) primers, the size of the amplified fragment (S), the template used for the reaction (T), and the annealing temperature (A) for the PCR were as follows. (i) IS*1400* (this study) SP, 5′-CACGCAGTTTACGATGAG-3′; ASP, 5′-GATTTCCAGTTTTTCACC-3′ (S, 787 bp; T, Ye 8081 genomic DNA; A, 53°C); (ii) IS*1328* (35) SP, 5′-CCAGTTAGGGCATCAGGTCA-3′; ASP, 5′-GTCGCAAA-ACCAGAAAGTCA-3′ (S, 872 bp; T, Ye 8081 genomic DNA; A, 56°C); (iii) insertion sequence downstream of the *yopE* gene (14) SP, 5′-TAAAACG-CCTGATGAACT-3′; ASP, 5′-ACGGATTTGTCTTTCTGG-3′ (S, ≈1 kb; T, Ye 8081 genomic DNA; A, 52°C); (iv) IS*100* (31) SP, 5′-ATTGATCCACCGTTTTACTC-3′; ASP, 5′-CGAACGAAAGCATGAAACAA-3′ (S, 963 bp; T, 6/69M genomic DNA; A, 52°C); (v) IS*200* (40) SP, 5′-CCAATGAGGGATGAAAAGAG-3′; ASP, 5′-AGTTGCTCACCCATTTTATC-3′ (S, 419 bp; T, 6/69M genomic DNA; A, 52°C); (vi) IVS (41) SP, 5′-AGATGTAACGGGGCTAAA-3′; ASP, 5′-TCACCTTCAACGGCTTAC-3′ (S, 180 bp; T, Ye 8081 genomic DNA; A, 52°C). The reaction mixtures were incubated with 0.25 U of *Taq* polymerase (United States Biochemical Corp.) in a PHC-3 thermocycler (Techne) for 30 cycles (denaturation, 94°C for 1 min; annealing, temperature as specified for each primer for 1 min; extension, 72°C for 1 min).

Nucleotide sequence accession numbers. The nucleotide sequence of the IS*1400* element with its surrounding region and that of the *asn* gene of *Y. enterocolitica* have been submitted to EMBL under accession numbers X94452 and X95298, respectively.

RESULTS

Physical map and dimensions of the HPI of *Y. enterocolitica* Ye 8081. To define the HPI of *Y. enterocolitica* Ye 8081, a chromosomal library of the 70-kb virulence plasmid cured strain (pYV$^-$) was constructed in the cosmid vector pHC79. Two colonies carried recombinant cosmids that hybridized either with both the E6 and Cl8 probes for *irp2* (pHC15) or with the E6 probe only (pHC25). Insert sizes were approximately 40 and 38 kb for pHC15 and pHC25, respectively. Previously, screening of several strains of biotype 1B or of serially subcultured individual colonies from strain Ye 8081 had failed to yield a spontaneous isogenic *irp2* deletion mutant (10). Accordingly, the HPI was defined as the region of DNA surrounding the *irp2* gene that was present in the high-pathogenicity strain Ye 8081 but absent from the low-pathogenicity *Y. enterocolitica* strains IP 864 and IP 383 (Table 1). Probes hybridizing only with strain Ye 8081 were considered to be inside the HPI, and those hybridizing with all three strains were considered to be outside the HPI. Some of the cosmid probes shown in Fig. 1A recognized not only the corresponding re-

striction fragment but also additional bands of various sizes, indicating that several repeated sequences (RS) are located on the HPI. Nevertheless, the probes were large enough to include sufficient nonrepeated conserved sequence to clearly distinguish the unique homologous fragment (strong hybridization signal) from the fragments carrying the repeated sequences (fainter hybridization signal). This procedure indicated that the HPI of strain Ye 8081 extends over approximately 45 kb. Its physical map is shown on Fig. 1A. Curiously, the *Hin*dIII, *Bgl*II, and *Bam*HI restriction sites were confined to a 10-kb region at the left-hand end of the HPI, while the remainder of the island contained *Eco*RI sites but no *Hin*dIII or *Bam*HI sites and only a single *Bgl*II site. The possibility of cosmid chimerism could be eliminated because (i) the probes which overlap the pHC15 cosmid were previously cloned into plasmids from Ye 8081 chromosomal DNA, (ii) the restriction map of the region extending from the *irp2* gene up to RS.2 was identical to that described for another strain of biotype 1B (35), and (iii) identical restriction sites were found in a cosmid different from pHC25 which covered the region between RS.2 and the left boundary of the HPI (Fig. 1A).

Characterization of the right boundary of the HPI. By the strategy described above, the right-hand border of the HPI of *Y. enterocolitica* Ye 8081 was identified on the EBa4 fragment (Fig. 1A). Hybridization using the entire EBa4 insert produced three *Eco*RI restriction fragments from the genome of strain Ye 8081, indicating that a repeated sequence is present on this fragment. To define the boundary more precisely, smaller probes were generated from the EBa4 fragment (Fig. 1B). The results of the hybridization experiments demonstrated that the right-hand border of the HPI is located on the 800-bp *Cla*I-*Hin*cII fragment (Fig. 1B). Confirmation that the HPI did not extend beyond this region was obtained by showing that the 5-kb *Bam*HI-*Bgl*II probe located to the right of the EBa4 segment hybridized with DNA from both high- and low-pathogenicity strains (data not shown). Furthermore, the fact that the 800-bp probe hybridized with the same three *Eco*RI restriction fragments as the entire EBa4 insert indicated that the RS present on this fragment is located at the right border of the HPI, on the *Cla*I-*Hin*cII fragment (Fig. 1B).

Sequencing from the *Bam*HI site of the EBa4 fragment revealed that the 124-bp region starting at the *Hin*cII site and extending toward the *Cla*I site (Fig. 1B) had a high degree of identity to the *asnT* gene encoding the tRNA-Asn of *E. coli* (22). The promoter region contained the −35 and −10 consensus regions characteristic of *tRNA* genes (Fig. 2A). It also displayed a typical stringency control discriminator element present in all *E. coli* genes under stringent control (15). As predicted, the rightmost element of the dyad overlaps the stringency control region. The structural *tRNA* gene of *Y. enterocolitica*, starting at nucleotide (nt) 49, was almost identical to the *E. coli* gene, with only one nucleotide mismatch. It contained the expected Asn anticodon (GTT) and the common CCA sequence which occurs at the 3′ terminus of all tRNAs (15). The predicted secondary structure of the tRNA-Asn of *Y. enterocolitica* obtained with the Staden program (43) displayed the characteristic cloverleaf structure of tRNA (Fig. 2B). Confirmation that the RS present on the 800-bp segment was indeed the *tRNA* gene was obtained by using a 50-bp internal oligonucleotide probe (nt 54 to 103 on Fig. 2A). This probe recognized three *Eco*RI restriction fragments in strain Ye 8081 (Fig. 3). Southern hybridization studies with the 50-bp probe also revealed that the *asn-tRNA* gene is present in a single copy on the HPI of strain Ye 8081, is absent from its pYV (not shown), and is present in two to five copies in the various species of *Yersinia* (Table 1).

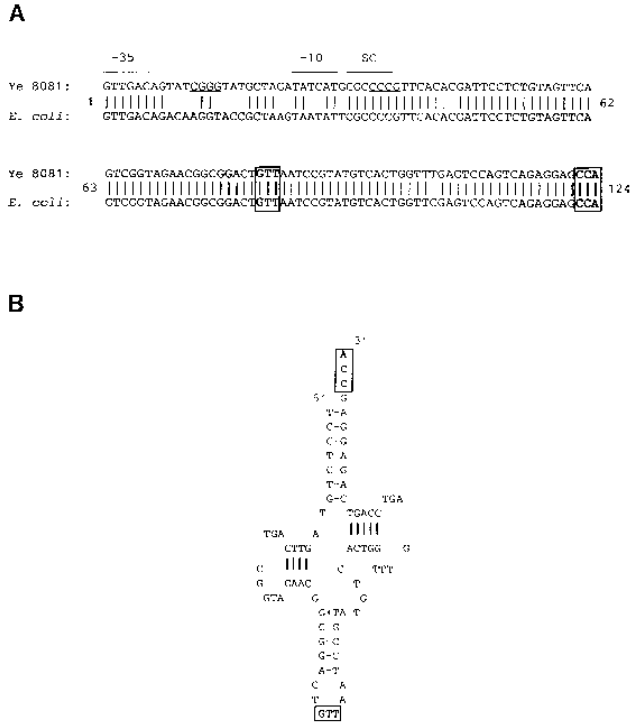

FIG. 2. (A) Nucleotide sequence of the region homologous to the *asn-tRNA* gene of *E. coli* (right boundary of the HPI of strain Ye 8081). −35 and −10, promoter sequence typical of the tDNA promoters; SC, stringency control discriminator element. Sequence elements with dyad symmetry are underlined. Nucleotides in boxes and bold type represent the Asn anticodon (GTT) and the 3′ terminus identical in all tRNAs (CCA). (B) Hypothetical tRNA folding obtained with the Staden program.

Characterization of the left boundary of the HPI. Similarly, the left-hand border of the HPI was identified on the HBg3.4 fragment (Fig. 1A), and smaller probes were generated to define it more precisely. The hybridization data summarized in Fig. 1C indicate that the left boundary of the HPI of strain Ye 8081 is located in the 0.4-kb *Xho*I-*Afl*II fragment. Confirmation that the HPI did not extend beyond the *Xho*I-*Bgl*II fragment was obtained by demonstrating that the 7-kb *Bgl*II-*Eco*RI segment located on the left side of the putative border hybridized with the DNA of both low- and high-pathogenicity strains (data not shown). Furthermore, the fact that the different DNA probes shown in Fig. 1C hybridized with only one *Eco*RI fragment indicates that, in contrast to the right-hand boundary, the left-hand boundary of the HPI is not a repetitive element.

Characterization of RS.1. One of the repeated sequences, named RS.1, was identified on the EBg2.4 fragment (Fig. 1A). This fragment hybridized with at least four *Eco*RI bands in strain Ye 8081 (Fig. 3). Sequencing of the 1,573-bp EBg2.4 fragment identified two major overlapping open reading frames (ORFs) located on the same DNA strand but on two different frames (Fig. 4): ORF.A (403 bp) and ORF.B (1,215 bp).

In ORF.A, a potential promoter region was identified 7 bp upstream of a putative Shine-Dalgarno ribosome binding site (Fig. 4). The initiation codon would then be the second ATG of ORF.A, and the corresponding product would be 88 amino acids (AA) long. A search of the European Molecular Biology Laboratory (EBI) protein database indicated that the predicted protein from ORF.A displayed the highest degree of similarity (67% identity and 82% similarity over an 87-AA

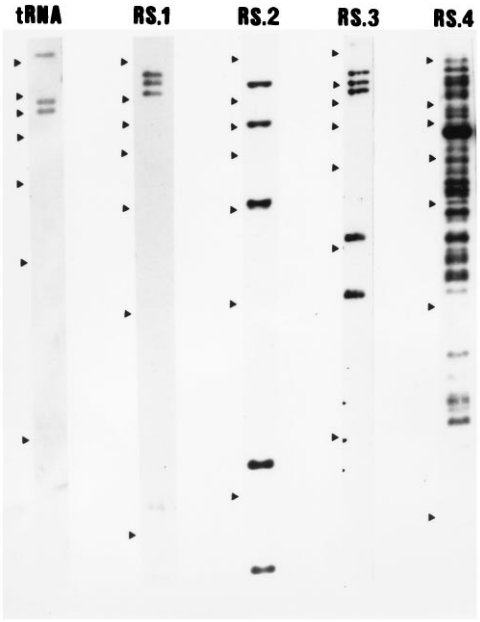

FIG. 3. Southern hybridization of the five repeated sequences present on the HPI with the *Eco*RI-digested genomic DNA of strain Ye 8081. The different probes used were a 50-bp-long oligonucleotide for the *asn-tRNA* gene (see Results), the 787-bp-long PCR product for RS.1 (described in Materials and Methods), and the E2.2, BCl1.3, and SCl1 fragments for RS.2, RS.3, and RS.4 respectively (Fig. 1A). Arrows on the left side of the autoradiographs indicate the following molecular sizes (in kilobases) from top to bottom: 23.6, 12, 10, 8, 6, 4, and 2.

stretch) with the LcrS protein of *Y. pseudotuberculosis* (37). *lcrS* is the last ORF of the Lcr region of the *Y. pseudotuberculosis* pYV plasmid. This region belongs to the *virC* operon involved in the export of the Yop proteins (26). Neither the function of the *lcrS* locus nor its product has been identified. Significant homology of the predicted protein from ORF.A was also found with two insertion elements: ISR1 (hypothetical 10-kDa protein A3) of a *Rhizobium* sp. (34) (50% identity and 73% similarity over an 86-AA stretch) and IS*407* (ORF4) of *Pseudomonas cepacia* (46) (52% identity and 75% similarity over an 84-AA stretch).

ORF.B occurs in frame −1 with respect to ORF.A. A search of the EBI protein database indicated that the predicted protein from ORF.B shared the highest degree of similarity (38% identity and 81% similarity over a 269-AA stretch) with ORF.B of the IS*476* insertion sequence of *Xanthomonas campestris* (21) (Fig. 4). It was thus surprising to find no homology between the predicted proteins encoded by ORF.A of RS.1 and ORF.A of IS*476*. However, after the correction of the published data (33) was used to update the nucleotide sequence of IS*476* lodged in the database, the two predicted proteins displayed 47% identity and 76% similarity over a stretch of 85 AA (Fig. 4). Significant levels of similarity of the predicted protein from ORF.B were also found with numerous other insertion elements. The highest scores were obtained for IS*407* (ORF1) of *P. cepacia* (46) (50% identity and 87% similarity over a 159-AA stretch) and IS*150* (ORF.B) of *E. coli* (39) (30% identity and 68% similarity over a 226-AA stretch).

All of these results indicate that RS.1 is an insertion element (registered under the designation IS*1400* at the Plasmid Reference Center in Stanford) and it belongs to the IS*3* family. The members of this family contain two consecutive and partly overlapping ORFs on the same strand with a repetition of at least six adenosine residues at the 3′ end of ORF.A, which induces a translational frameshifting, resulting in the synthesis of a transposase fusion protein (32). Like the other members of this family, IS*1400* is composed of two consecutive overlapping ORFs with a typical string A6A at the 3′ end of ORF.A (Fig. 4). The resulting fusion protein, which might be the transposase of IS*1400*, would be 370 AA long (≈40.7 kDa) with a pI of 9.92. The IS*3* family of insertion elements is also characterized by the existence of terminal imperfect repeats flanked by a duplication of 2 to 4 bp in the target DNA. A 16-bp-long perfect inverted repeat, followed by a 26-bp imperfect repeat, also flanks the IS*1400* nucleotide sequence, generating a 2-bp (TT) duplication in the target DNA (Fig. 4).

Strikingly, a search of DNA sequence databases revealed significant homologies between different portions of ORF.A and/or ORF.B and intergenic regions of the *Yersinia* pYV virulence plasmid. In *Y. enterocolitica*, such homologies were found in the DNA sequences located upstream of *yadA* (81% identity over 90 nt) (42) and between *yop20* (*yopQ* in the new nomenclature) and *ylpA* (72.5% identity over 280 nt) (27). On the *Y. pseudotuberculosis* plasmid, a sequence sharing some homology (59% over 267 nt) with IS*1400* was identified between *yopJ* and *yopH* (16, 23). These results suggest that the short regions of homology found in the intergenic regions of the pYV plasmid may be scars of ancient insertions.

The copy number of IS*1400* was studied by Southern hybridization with a 787-bp-long internal DNA fragment obtained after PCR amplification (see Materials and Methods). Only one copy of IS*1400* is located on the HPI, and at least three other copies of this insertion sequence are present elsewhere on the chromosome of strain Ye 8081 (Fig. 3). In addition, three *Eco*RI restriction fragments of approximately 20, 14, and 5.5 kb were recognized on the pYV of this strain (data not shown). These hybridizing fragments may correspond either to the presence of a complete IS*1400* at different sites on the pYV or more probably, to the partial homology found between IS*1400* and the intergenic sequences adjacent to *lcrS*, *yadA*, *yopQ*, or *yopH*. Copies of IS*1400* are also present in several pathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis* but are absent from *Y. pestis* and all the nonpathogenic *Yersinia* species tested (Table 1).

Identification and distribution of RS.2. A second repeated sequence, RS.2, was identified on the E2.2 fragment of the HPI (Fig. 1A). This fragment hybridized with five *Eco*RI restriction fragments from the pYV-strain Ye 8081 (Fig. 3). The position of the *Eco*RI sites relative to the *irp2* gene and the copy number of RS.2 strongly suggested that RS.2 was the recently described insertion sequence IS*1328* located downstream of the *fyuA* locus (35). To confirm this hypothesis, two primers internal to the gene encoding IS*1328* were synthesized (see Materials and Methods) and used to amplify by PCR an 872-bp DNA fragment that was hybridized with the *Eco*RI-digested cosmid pHC25. The probe recognized the expected 2.2-kb *Eco*RI fragment carrying the RS.2 sequence (data not shown), confirming that RS.2 is the IS*1328*. This result also allowed us to position the *fyuA* gene on the restriction map of the HPI (Fig. 1A). Hybridization studies with the amplified 872-bp fragment revealed that there is only one copy of IS*1328* on the HPI and that another copy is present on the pYV of strain Ye 8081. The distribution of IS*1328*, determined with the E2.2 probe, among different *Yersinia* species (Table 1) indicates that this insertion sequence is widely distributed in the genus *Yersinia* and that its presence is not linked to the level of pathogenicity of the strains.

Distribution of RS.3. Another repeated sequence, called RS.3, between RS.1 and RS.2 was identified on a 1.3-kb

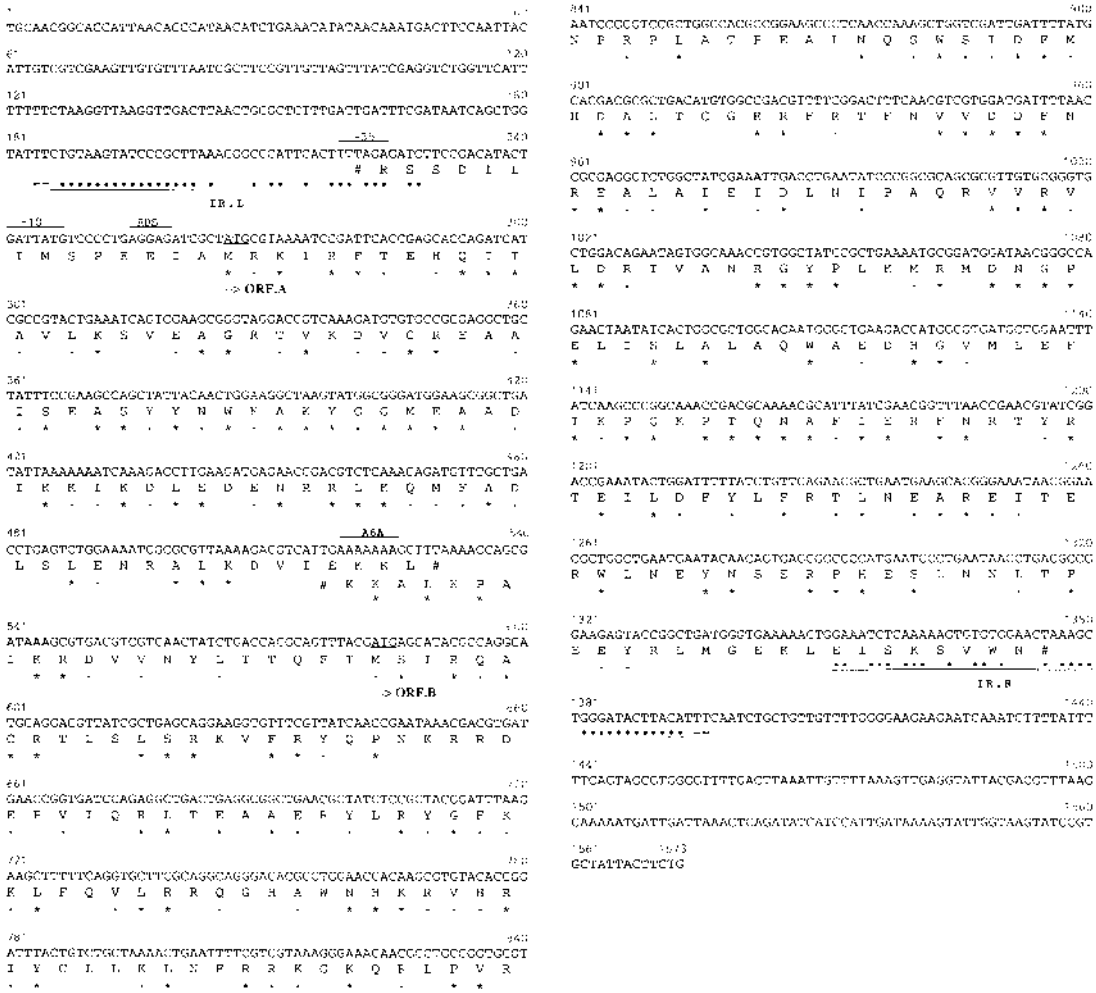

FIG. 4. Nucleotide and amino acid sequences of the EBg2.4 insert encompassing the IS*1400* insertion element. −10 and −35, promoter sequence; RBS, ribosome binding site; IR-L and IR-R, left and right imperfect terminal inverted repeats; ·, identical nucleotides in the two repeats; ==, target site duplication; A6A, stretch of polyadenosine where ribosomal rephrasing may occur; ∗, identical amino acids; ., conserved amino acids between the RS.1 and IS*476* predicted protein sequences.

*Bam*HI-*Cla*I fragment (BCl1.3), and it slightly overlapped the *Cla*I site (Fig. 1A). Hybridization studies with the BCl1.3 probe revealed that only one copy of RS.3 is present on the HPI but that at least four additional copies are located elsewhere on the chromosome of strain Ye 8081 (Fig. 3) and none are on the virulence plasmid. The distribution of RS.3 among different *Yersinia* species (Table 1) indicates that RS.3 is present in variable copy numbers (1 to 5) in all *Y. enterocolitica* 1B species but that its presence in other *Yersinia* species is not linked to their degree of pathogenicity.

Distribution of RS.4. Finally, a fourth repeated sequence, designated RS.4, was identified immediately upstream of the *irp2* gene (Fig. 1A). The 1-kb *Sph*I-*Cla*I fragment (SCl1) overlapping the RS.4 sequence was obtained from the cloned Cl8 segment and used as a probe for Southern hybridization. RS.4 is present in multiple copies (>20) on the chromosome of strain Ye 8081 (Fig. 3), but only one copy is located on its HPI. A faint 11-kb *Eco*RI restriction fragment was recognized by the SCl1 probe on the pYV plasmid after long exposure times, suggesting that either some cross-hybridizing sequences or a short segment of RS.4 might be present on this plasmid. Numerous copies of RS.4 were found in all the different strains and species of *Yersinia* tested (Table 1), indicating that this RS is widely spread in the genus *Yersinia.*

Search for the presence of known repeated sequences on the HPI of strain Ye 8081. At least five repeated sequences have previously been reported among pathogenic *Yersinia* species: (i) the IS*100* that flanks the HPI of *Y. pestis* (13), (ii) the IS*200*-like element which lies within the *inv* gene of *Y. pestis* (40), (iii) the IS*3*-like element identified downstream of the *ail* gene and specific for high-pathogenicity strains (28), (iv) the insertion sequence located downstream of the *yopE* gene of *Y. enterocolitica* (14), and (v) an approximately 100-bp intervening sequence (IVS) that interrupts the 23S ribosomal DNA of pathogenic *Y. enterocolitica* (41). We wondered whether one of these characterized RS might correspond to RS.3 or RS.4. To test this hypothesis, two approaches were used. In the case of the IS*3*-like element adjacent to the *ail* gene, the *ailB* fragment carrying this element (28) was extracted from the pVM103 plasmid kindly provided by S. Falkow and J. Bliska (Stanford) and used as a probe. For the other repeated sequences, primers were designed on the basis of published nucleotide sequences (see Materials and Methods) and the corresponding fragments were amplified by PCR. The eluted *ailB* fragment

Strains	Probes: Serotype	Bg7 (*Bgl*II)#	EBg2.4	X	BaE2	E22	E3.5	E8.8	E6	E9	EBa4
Ye 8081	O:8	15 (7)	15**	36	3.6**	22**	3.5	8.8	6	9**	23**
WA	O:8	= (=)	=**	39	3.9**	=**	=	=	=	=**	=**
IP 1105	O:8	= (=)	=**	39	3.9**	=**	=	=	=	=**	=**
IP 24763	O:8,13	14 (=)	**	41	4.1**	**	=	=	=	=**	=**
IP 24764	O:13,20	11.5 (>23)	=**	-	**	**	=	=	=	=**	=**
IP 24766	O:20	14 (=)	**	41	4.1**	**	=	=	=	=**	=**
IP 845	O:20	14 (=)	**	41	4.1**	**	=	=	=	=**	=**
IP 24762	O:21	10.5 (>23)	**	-	**	=**	=	=	=	=**	15**
IP 1110	O:21	14 (=)	**	41	4.1**	**	=	=	=	=**	=**
IP 24761	O:4,32-4,33	= (=)	=**	=	=**	=**	=	=	=	=**	=**

FIG. 5. Comparison of the physical map of the HPI of strain Ye 8081 with those of nine other *Y. enterocolitica* strains of biotype 1B. Arabic numbers indicate sizes of the hybridizing fragments. X, EBa1.2 probe; =, hybridizing fragments with a size similar to that of strain Ye 8081; **, several hybridizing fragments; =**, fragment with a size similar to that of strain Ye 8081 plus additional fragments; −, no hybridizing fragment; #, size of the hybridizing fragments when the DNA was digested with *Bgl*II instead of *Eco*RI.

and the PCR products were used as probes for Southern hybridization with the *Eco*RI-digested DNA of cosmids pHC15 and pHC25. The results of the hybridization experiments indicated that none of the previously identified RS of *Yersinia* spp. was located on the HPI of strain Ye 8081 (data not shown). Therefore, RS.3 and RS.4 are novel repeated sequences in *Yersinia* spp.

Conservation of the HPI among biotype 1B *Y. enterocolitica* strains. In a previous study, we found that the hybridization pattern of the *irp2* gene was identical in all *Y. enterocolitica* 1B strains tested (10), indicating that this portion of the HPI was well conserved in this bacterial group. We wondered whether the whole length of the HPI was also conserved among *Y. enterocolitica* 1B. To answer this question, genomic DNA prepared from the 10 strains of biotype 1B listed in Table 1 was digested with *Eco*RI and hybridized with 10 probes that covered the entire length of the HPI of strain Ye 8081 (Fig. 5). The regions encompassing RS were difficult to analyze because the probes hybridized with several restriction fragments. However, in most cases, the probes contained enough unique nonrepeated sequence to increase the hybridization signal of one band and to thereby identify the homologous fragment. As shown in Fig. 5, the HPI region extending leftward from the right-hand boundary, up to the 3′ end of the *fyuA* gene, was identical in all biotype 1B strains tested. The smaller *Eco*RI fragment hybridizing with the EBa4 probe in strain IP 24762 (Fig. 5) was found to reflect a modification of the *Eco*RI site located outside the HPI. Similarly, the various sizes of the *Eco*RI fragments hybridizing with the Bg7 probe located at the left-hand side of the HPI reflected DNA variations outside the island. The only two exceptions were strains IP 24764 and IP 24762, whose *Bgl*II hybridizing fragment was larger than 23 kb. The main variations in the hybridization patterns were observed in the region of the HPI encompassing RS.1 to RS.3 (Fig. 5). On the basis of these results, the HPIs of the 10 biotype 1B *Y. enterocolitica* strains studied could be divided into four main categories. The first group was composed of strain Ye 8081 and a single serotype O:4,32-4,33 strain (IP 24761) whose HPIs were identical (Fig. 5). The second group was composed of the two other strains of serotype O:8 (WA and IP 1105), which showed conservation of the HPI throughout their whole length, except for the 3.6-kb *Eco*RI fragment carrying RS.3 (Fig. 5). In the third group, including four strains of serotypes O:20 (IP 845 and IP 24766), O:21 (IP 1110), and O:8,13 (IP 24763), the HPI differed from that of strain Ye 8081 in the 7.8-kb region encompassing RS.1 to RS.3. The fourth and most divergent group contained two strains of serotype O:13,20 (IP 24764) and O:21 (IP 24762). The identity between the hybridization patterns of strain Ye 8081 and this group stopped downstream of the *fyuA* gene, in the 15-kb left-hand part of the HPI (Fig. 5). Altogether, these results indicate that the HPI of Ye 8081 can be divided into two main regions: a 30-kb right-hand portion which is conserved in all strains studied and a 15-kb left-hand portion which exhibits strain-dependent variation.

DISCUSSION

The HPI of *Y. pestis* is a 102-kb chromosomal fragment which carries the genes encoding the pigmentation phenotype, the yersiniabactin-pesticin receptor, and the high-molecular-weight proteins (12). On screening by Southern blotting with cosmid subclones of this region, Fetherston et al. (13) found that only a single cosmid located at the right-hand side of the HPI of *Y. pestis* hybridized with the DNA of a *Y. enterocolitica* O:8 strain. This result suggested that most of the HPI of *Y. pestis* was absent from *Y. enterocolitica* either because this region was reduced in size in *Y. enterocolitica* or because part

of the HPI was less well conserved. In the present work, the HPI of strain Ye 8081 of *Y. enterocolitica* has been characterized. Our results indicate that the HPI of *Y. enterocolitica* is 45 kb long and therefore is indeed much smaller in size than that of *Y. pestis.*

Also in contrast to *Y. pestis*, the HPI of *Y. enterocolitica* 1B is not flanked by copies of IS*100*. Although an element repeated elsewhere on the chromosome is present at one boundary of the island, it is not present at the other extremity, and this sequence is not an insertion element but a copy of the *asn-tRNA* gene. In this respect, the HPI of *Y. enterocolitica* is closer to the pathogenicity islands (PAI I and II) of extraintestinal *E. coli* (18) and to the virulence-associated regions (*vap* regions) of *Dichelobacter nodosus* (9) than to the HPI of *Y. pestis*. In uropathogenic *E. coli*, a selenocysteine tRNA gene is present at one boundary of PAI I and a leucine tRNA gene is present at one boundary of PAI II (4). Deletion of the PAIs involves two different direct repeats of 16 (PAI I) or 18 (PAI II) nt located at each extremity of the PAIs. Each tRNA gene contains one of the two copies of the direct repeat close to its 3′ end. Following the deletion event, only one repeat remains at the junction site, and the resulting truncated tRNA gene becomes transcriptionally inactive (4). Similarly, one of the *vap* regions of *Dichelobacter nodosus* is bounded by two 19-bp sequences, and a *ser-tRNA* gene is found at one extremity of this region (9). Whether such a phenomenon is responsible for the spontaneous deletion of the HPI described for strain WA is not yet clear. It is tempting to speculate that this mechanism has also been responsible for the excision of the HPI in an ancestral strain of *Y. enterocolitica* and has led to the emergence of the low-pathogenicity group of *Y. enterocolitica* of biotypes 2 to 5. Further experiments will be necessary to confirm this hypothesis.

Another particular feature of the HPI of *Y. enterocolitica* is the presence of several different repeated sequences. In addition to the tRNA gene, at least four other repeated sequences are located on the island. We have identified one of these elements, RS.1, as a novel insertion sequence belonging to the IS*3* family and homologous to IS*476* of *X. campestris*. This new insertion sequence has received the designation IS*1400*. Although the genera *Yersinia* and *Xanthomonas* are not phylogenetically close, besides these two related insertion elements IS*1400* and IS*476*, they also share homologies in several genes encoding similar type III secretion systems (45), suggesting horizontal gene exchanges between the two genera. In *Yersinia* spp., the type III secretion system is used to export the plasmid-encoded Yop proteins (27). The fact that a DNA sequence homologous to ORF.A of IS*1400* is present on the virulence plasmid of *Yersinia* spp. may indicate that this mobile element had participated in the transfer of the type III secretion system between different bacterial genera.

The second repeated sequence identified on the HPI of *Y. enterocolitica*, RS.2, is the IS*1328* insertion element described by Rakin and Heesemann (35). These authors suggested that IS*1328* could be responsible for the sequential deletions of the *fyuA-irp2* gene cluster in the same way that IS*100* is thought to provoke deletion of the HPI in *Y. pestis*. However, we have found only one copy of IS*1328* in the HPI region of *Y. enterocolitica*, so recombination between flanking copies of this element is an unlikely cause for the deletion.

Strikingly, a pronounced clustering of insertion elements exists in the vicinity of PAI II on the *E. coli* K-12 map. It has been suggested that this region might influence the deletion of the neighboring pathogenicity island (4). In *Y. enterocolitica*, a clustering of repeated sequences downstream of the *fyuA* gene may indicate a cognate organization and suggests that this region could also play some role in the instability of the island. The fact that the conservation of the HPI of different high-pathogenicity *Y. enterocolitica* strains is interrupted in the region containing the cluster of repeated sequences reinforces this hypothesis.

In addition to the different repeated sequences, the genes identified so far on the HPI of *Y. enterocolitica* are (i) the *fyuA* locus which codes for the yersiniabactin-pesticin receptor and (ii) the *irp2* and possibly *irp1* loci that encode high-molecular-weight proteins involved in the nonribosomal synthesis of a small peptide which may be the siderophore yersiniabactin. Therefore, it seems that a large proportion of the HPI of *Y. enterocolitica* is devoted to iron capture. It is known that a crucial difference between high- and low-pathogenicity strains of *Y. enterocolitica* is their abilities to scavenge iron in vivo (38). The absence of the HPI in low-pathogenicity strains may thus explain their inability to chelate the iron molecules bound to host proteins and therefore their inability to cause invasive disease. Nevertheless, even if the presence of an iron capture system is essential for the expression of the high-pathogenicity phenotype, it is likely that other genes also participate to this phenotype. The presence of additional high-pathogenicity genes in this island or other islands elsewhere on the chromosome of *Y. enterocolitica* 1B strains remains to be investigated.

In conclusion, the high-pathogenicity island of strain Ye 8081 of *Y. enterocolitica* is a 45-kb chromosomal fragment which carries several repeated sequences and genes involved in iron capture. One extremity of the HPI is an *asn-tRNA* gene. This genomic organization, grouping genes into pathogenicity islands adjacent to *tRNA* genes, is an increasingly well-recognized phenomenon in bacterial pathogens. These islands may result from the insertion of extrachromosomal replicons carrying virulence genes into the bacterial genome. The use of *tRNA* genes as integration sites is shared by many different plasmids and bacteriophages (9). Whether these islands still possess the ability to excise from the genome and to be transferred to other bacterial species and what are the other genes carried by the HPI remain to be determined, but these questions represent an exciting and promising field of investigation.

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