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Common and Specific Characteristics of the High-Pathogenicity Island of *Yersinia enterocolitica*

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***Yersinia pestis*, *Y. pseudotuberculosis* O:1, and *Y. enterocolitica* biogroup 1B strains carry a high-pathogenicity island (HPI), which mediates biosynthesis and uptake of the siderophore yersiniabactin and a mouse-lethal phenotype. The HPI of *Y. pestis* and *Y. pseudotuberculosis* (Yps HPI) are highly conserved in sequence and organization, while the HPI of *Y. enterocolitica* (Yen HPI) differs significantly. The 43,393-bp Yen HPI sequence of *Y. enterocolitica* WA-C, serotype O:8, was completed and compared to that of the Yps HPI of *Y. pseudotuberculosis* PB1, serotype O:1A. A common GC-rich region (G+C content, 57.5 mol%) of 30.5 kb is conserved between yersinia strains. This region carries genes for yersiniabactin biosynthesis, regulation, and uptake and thus can be considered the functional "core" of the HPI. In contrast, the second part of the HPI is AT rich and completely different in two evolutionary lineages of the HPI, being 12.8 kb in the Yen HPI and 5.6 kb in the Yps HPI. The variable part acquired one IS*100* element in the Yps HPI and accumulated four insertion elements, IS*1328*, IS*1329*, IS*1400*, and IS*1222*, in the Yen HPI. The insertion of a 125-bp ERIC sequence modifies the structure of the promoter of the *ybtA* yersiniabactin regulator in the Yen HPI. In contrast to the precise excision of the Yps HPI in *Y. pseudotuberculosis*, the Yen HPI suffers imprecise deletions. The Yen HPI is stably integrated in one of the three *asn* tRNA copies in *Y. enterocolitica* biogroup 1B (serotypes O:8, O:13, O:20, and O:21), probably due to inactivation of the putative integrase. The 17-bp duplications of the 3′ end of the *asnT* RNA are present in both *Yersinia* spp. The HPI attachment site is unoccupied in nonpathogenic *Y. enterocolitica* NF-O, biogroup 1A, serotype O:5. The HPI of *Yersinia* is a composite and widely spread genomic element with a highly conserved yersiniabactin functional "core" and a divergently evolved variable part.**

The genus *Yersinia* consists of 11 species. Strains of *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are pathogenic for mammals. Pathogenicity determinants have been localized on plasmids and on the chromosome of yersiniae. Pathogenic *Yersinia* can be divided into a high-pathogenicity group (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* biogroup [BG] 1B) and a low-pathogenicity group (*Y. enterocolitica* BG 2 to 4), on the basis of the lethal infectious dose in the mouse model (9). Lethality for mice (50% lethal dose < 1,000 microorganisms) depends on the presence of the yersiniabactin (*ybt*) locus, which carries genes for biosynthesis, transport, and regulation of the siderophore yersiniabactin (22–24, 28, 31). In *Y. pestis*, the unstable 102-kb chromosomal fragment is associated with pigmentation (*pgm*), i.e., the ability of bacterial cells to form pigmented colonies on hemin or Congo red agar plates at 26°C (31). The *pgm* locus is composed of yersiniabactin (*ybt*) and hemin storage (*hms*) loci (15, 25, 27, 29). In contrast, *Y. pseudotuberculosis* contains nonclustered *ybt* and *hms* loci and only the *ybt* locus is present in *Y. enterocolitica* (5, 8). The in vitro instability of the *pgm* locus in *Y. pestis* has been observed as a complete or partial (*hms* or *ybt* locus) deletion of the 102-kb fragment due to the presence of two IS*100* flanking sequences (12, 14, 15). The *ybt* locus comprises 36 to 43 kb (8). Sequencing of genes involved in yersiniabactin synthesis and uptake revealed a G+C content higher than that of the host genome (21, 36). The *ybt* locus is flanked by an *asn* tRNA gene at one extremity and carries a gene for a putative integrase (4, 8). These are features typical of pathogenicity islands (20). Therefore, the *ybt* locus is termed a high-pathogenicity island (HPI) to emphasize its involvement in the mouse-lethal phenotype (8). Interestingly, the *ybt* cluster has been detected in certain pathotypes of *Escherichia coli*, suggesting that it originates from a horizontal transfer (40).

Five synthesis genes, *irp1* to *irp5* (*ybtU*, *ybtT*, and *ybtE* are the orthologs of *irp3*, *irp4*, and *irp5* in *Y. pestis*) of the *ybt* locus are clustered in one large 19-kb operon (2, 28). The genes for the yersiniabactin receptor FyuA (also named Psn in *Y. pestis*) and an AraC-type yersiniabactin regulator YbtA flank the biosynthetic genes (16, 17, 33). An RS3 repeated sequence and two IS elements were identified downstream of *fyuA* in *Y. enterocolitica* (8, 34).

Two distinct variants of the HPI were identified in *Y. pseudotuberculosis*/*Y. pestis* (Yps HPI) and in *Y. enterocolitica* (Yen HPI) (36). The part of the island that contains yersiniabactin synthesis, receptor, and regulator genes is highly conserved in HPIs of both evolutionary lineages (28, 36), while the right end (downstream of *fyuA*) differs markedly between the Yps HPI and the Yen HPI (5, 8). The Yps HPI is able to occupy any of the three asparagine tRNA genes in *Y. pseudotuberculosis*, suggesting that the HPI has retained its mobility functions (4). The HPI is flanked by 24-bp (21) or 17-bp (4) direct repeats that are duplications of the 3′ end of *asn* tRNA.

In this work, we determined the complete molecular genetic structure of the HPI in *Y. enterocolitica* WA-314 and compared it with that of the *Y. pseudotuberculosis* HPI to gain insight into the divergent evolution of the HPI and *Yersinia* in general.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in the study are listed in Table 1. The strains were grown in Luria-Bertani (LB) broth or on LB agar plates (Difco Laboratories, Detroit, Mich.) at 28°C (*Yer-*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Y. pestis		
KIM	BG mediavalis	R. R. Brubaker
KIM *pgm*	Spontaneous nonpigmented mutant of KIM	This study
Y. pseudotuberculosis		
PB1	Serotype O:1A	R. R. Brubaker
PB1 Nal^r	Nal^r derivative of PB1	This study
Y. enterocolitica		
WA-314	Clinical isolate, O:8 BG 1B	23
WA-C	Plasmidless derivative of WA-314, Nal^r	23
8081	Clinical isolate, O:8 BG 1B	32
Ye1209-79	Clinical isolate, O:13 BG 1B	23
Ye1223-75-1	Clinical isolate, O:20 BG 1B	23
Ye737	Clinical isolate, O:21 BG 1B	23
Y-108	Clinical isolate, O:3 BG 4	23
108-C	Plasmidless derivative of Y-108	23
H567/90	Clinical isolate, O:5,27 BG 3	23
Y-96	Clinical isolate, O:9 BG 2	23
96-C	Plasmidless derivative of Y-96	23
NF-O	Clinical isolate, O:5 BG 1A	37
E. coli		
Phi	Pesticin sensitive, carrying HPI	R. R. Brubaker
K49	Pesticin sensitive, carrying HPI, ColK, O:156	36
K235	Pesticin sensitive, carrying HPI, ColK, O:1	36
D-1041-86	Enteroaggregative, O:44	40
C-4441	Enteroaggregative, O:128	40
12860	Enteroinvasive, O:124	40
Plasmids		
12H2	pLAFR2 cosmid carrying *Y. enterocolitica* WA-C HPI sequences upstream of the *irp2* gene, Tc^r	35
D11	pLAFR2 cosmid carrying *Y. pseudotuberculosis* PB1 HPI sequences downstream of the *irp1* gene	36
pRS3	*Eco*RI-*Bam*HI fragment of 8081 HPI carrying RS3	8
pEBa	*Bam*HI-*Eco*RI fragment of 8081 between pRS3 and EBg2.4	8
EBg2.4	*Eco*RI-*Bgl*II fragment of 8081 HPI carrying IS*1400*	8
pMOS	Cloning vector, Ap^r	Amersham
pBluescript KSII	Cloning vector, Ap^r	Stratagene

sinia) or 37°C (*E. coli*). Iron-chelating compounds were screened on a chrome azurol S ferric ion indicator dye (CAS) agar (41). A red-orange halo around bacterial colonies indicated siderophore production (i.e., colonies were CAS agar positive). *Y. pestis* spontaneous mutants, unable to accumulate the Congo red dye (*pgm*), were selected on LB medium containing 15 μg of Congo red per ml.

DNA manipulations. Bacterial DNA was isolated by the method of Davis et al. (11). A *Y. enterocolitica* gene bank was prepared from WA-314 serotype O:8, and cosmid 12H2 was used to determine the 5′ end of the HPI (35). The D11 cosmid of the *Y. pseudotuberculosis* PB1 serotype O1A gene bank was used to determine the 3′ end of the HPI (36). PCR amplifications were performed in an automated thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer) as described by Saiki et al. (39) with *Taq*I polymerase and different pairs of oligonucleotides (Roth, Karlsruhe, Germany, and Metabion, Munich, Germany). Self-designed PCR primers used to define the ends of HPI and to amplify specific HPI sequences are listed in Table 2. Southern blot hybridizations were performed with digoxigenin (DIG)-labeled PCR probes, using different primer pairs plus DIG–11-dUTP, as specified by Boeringer Mannheim Biochemica.

DNA sequencing and sequence comparison. Cosmid 12H2 (35) was used to determine sequences located between *ybtA* and the left end of the island associated with *asn* tRNA. Four *Cla*I fragments of the 12H2 cosmid subcloned in pBluescript KSII cloning vector (Stratagene), and the original 12H2 cosmid was used to determine the sequence of the 5′ boundary of the HPI by primer walking.

The sequence between *fyuA* and the 3′ end of the Yps HPI was determined by a combination of subcloning and primer walking with the *Y. pseudotuberculosis* O:1A cosmid D11, which contains sequences downstream of *fyuA* (36). Four *Eco*RI and three *Pst*I fragments of the D11 cosmid subcloned in pBluescript KS II vector, and the D11 cosmid itself were sequenced to establish the 3′ boundary of the island.

Primers 3P345 (corresponding to the right junction of the island) and 3P6057 (which resides in the region downstream of the HPI that is similar in both evolutionary lineages) amplified a sequence downstream of the pathogenicity island in *Y. enterocolitica* WA-C.

The sequence between IS*1328* and IS*1400* was determined in *Y. enterocolitica* 8081 with the three subclones pRS3 (containing IS*1329* and partial IS*1222* insertion sequences), pEBa (containing sequences between pRS3 and EBg2.4), and EBg2.4 (carrying the IS*1400* mobile element) (8). Plasmids pRS3, pEBa, and EBg2.4 were a kind gift from E. Carniel, Institute Pasteur, Paris, France. Sequencing of the PCR fragments obtained with *Y. enterocolitica* 8081 chromosomal DNA confirmed the junctions between the subcloned fragments. Bearing in mind the high identity of the *Y. enterocolitica* WA-C and 8081 sequences (8), the primers designed for the HPI in strain 8081 were used to determine the sequence in strain WA-C. The 5.3-kb DNA fragment between IS*1400* and the 3′ end of the Yen HPI was obtained in *Y. enterocolitica* WA-C by a PCR with the Ye9765 (located downstream of IS*1400*) and Ye262 (positioned downstream of the right direct repeat DR_{17} flanking the HPI) primers.

DNA sequencing was performed by the chain termination method with a model ABI 377 DNA sequencer (ABI Prism; Perkin-Elmer). Alignment and sequence comparison were performed with the HIBIO Mac DNASIS (Hitachi Software Engineering Co.) and DNAMAN (Lynnon BioSoft) programs and with the sequence analysis software package of the Genetics Computer Group (University of Wisconsin, Madison, Wis.). The island was analyzed for the presence of open reading frames (ORFs) containing at least 100 codons.

BLAST searches were performed on the NCBI server (27a). HPI sequences were also compared with the *Y. pestis* CO-92 sequences presented by the *Y. pestis* Sequencing Group at the Sanger Centre (39a).

Since there is no consensus on uniform nomenclature, we have used the *irp* (iron-repressed proteins [8]) designation for the genes located on the HPI. The orientation of the genes on the HPI is the same as proposed by Fetherston and

TABLE 2. Primers used in this study

Primer	Primer sequence (5′ to 3′)	Orientation
Integrase gene		
int520	ACATCCTTGCGAATCCTTATC	Forward
c15-205	TACAGGCAGGTTCCCGATGAC	Reverse
int1597	CCTGTGGAGGTGGTGGTAAT	Reverse
ybtA promoter		
ybt7524	AAACAGGGTCGGGAGAGGATT	Forward
D7	GGACATCGATTCAGTATTGGA	Forward
ybt1012	GCCATAGACGCTGTTGTTGAA	Reverse
D340	GGATTGCGTTTGCGGTGACTC	Reverse
3′ end of Yen HPI		
HPI878	GGGGGCAAGAAAAACTAACC	Forward
HPI387	TCATTAAATAGTCACCCCATAG	Forward
Ye9765	CAAGTGTCACCAGCGTAGGGATTTC	Forward
HPI1220	TTTGTTTTATGGCTTTGGTAG	Reverse
HPI283	TTTCAGTGTTTCAGCGTCCAG	Reverse
3P6057	CAACTTGAGCGTGATAAACA	Reverse
Left junction of Yen HPI		
W250	TCGGCTCGACGCTTTAGGTAG	Forward
W598	CCAATTTTTCCCGATGCGTAG	Reverse
Right junction of HPI		
3P345	GAGGCGATCCCAGTCAGAG	Forward
Ye262	TTTTCCCCCGAGAGGCTGAGTAACC	Reverse for Yen HPI
W36878	TGGCTTCCCCGCTTTTTATCGTAT	Reverse for Yps HPI
asn tRNA gene		
asn468	CCGTATGTCACTGGTTCG	Forward

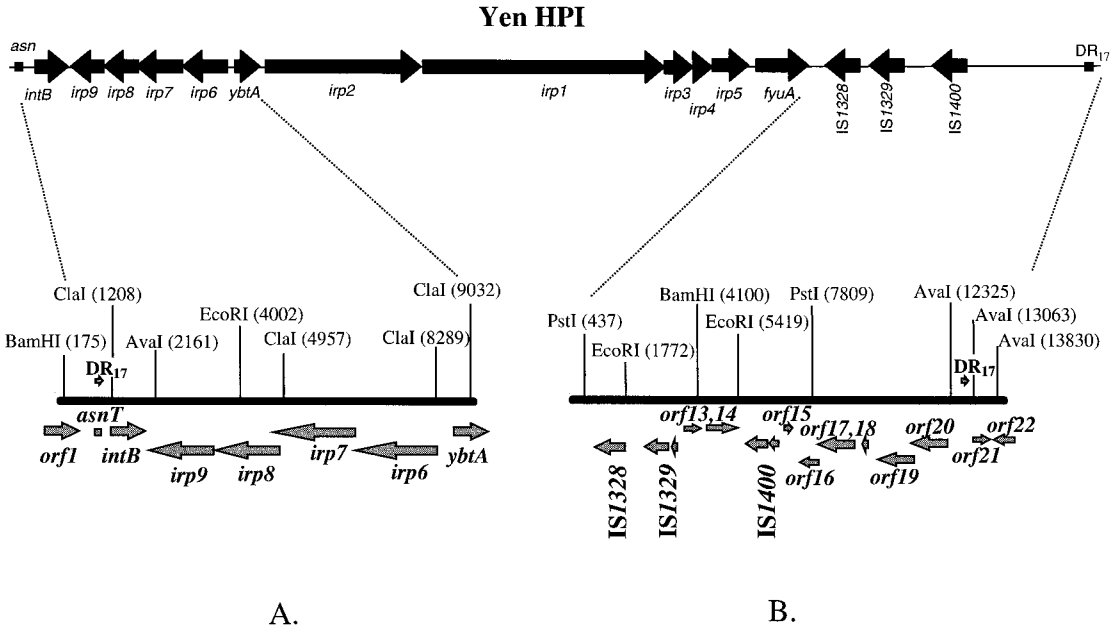

FIG. 1. Complete structure of the HPI in *Y. enterocolitica* WA-C. The Left (5′) (A) and right (3′) (B) ends of the island are shown. Arrows show the positions of the ORFs and the direction of transcription. Positions of restriction sites are depicted by vertical lines; numbers above the lines show the distance from the beginning of the sequenced DNA fragment in base pairs.

Perry (15). We have defined that the *intB* gene adjacent to the *asn* tRNA bacterial attachment site is at the 5′ or left extremity of the HPI and that the *fyuA* gene resides at the 3′ or right extremity of the island.

Nucleotide sequence accession numbers. The sequences determined in this study were deposited at the EMBL/GenBank database under accession no. AJ132668, AJ132945 and AJ236887.

RESULTS

To complete the structure of the Yen HPI in *Y. enterocolitica* WA-C, serotype O:8, we sequenced both ends of the island (Fig. 1). The borders of the island can be defined by 17-bp direct repeats flanking the HPI. The Yen HPI is 43,393 bp. The left (5′) end, which is associated with the *asn* tRNA gene, is nearly identical to that of the Yps HPI, while the right (3′) end shows significant differences between the *Y. enterocolitica* O:8 HPI and the Yps HPI (5, 8). Therefore, the DNA sequence of the 3′ end of the HPI in *Y. pseudotuberculosis* PB1, O1A (Yps HPI), was also determined and compared to the 3′ end of the Yen HPI. The comparison of Yen and Yps evolutionary lineages of the HPI reveals that the HPI has two distinct parts (Fig. 2). The first part spans the region between *asn* tRNA and the *fyuA* stop codon and represents the functional "core" of the island. It is nearly identical (98 to 99% identity) in both evolutionary lineages and has a G+C content significantly higher than that of the *Yersinia* genome (57.5 mol% versus 46 to 48 mol% for the *Yersinia* chromosome) (3). A considerably lower G+C content is found downstream of the *fyuA* gene. This AT-rich variable part differs completely between the HPIs of the two evolutionary lineages.

Left (5′) end of the HPI in *Y. enterocolitica*. The nucleotide sequence of the 8.1-kb fragment at the 5′ end of the Yen HPI contains five ORFs flanked by *ybtA* and *asn* tRNA (Fig. 1A). Four ORFs, *irp6* to *irp9*, have the opposite transcriptional polarity to *ybtA*. These four genes display 98 to 99% identities to the *ybtP*, *ybtQ*, *ybtX*, and *ybtS* genes, respectively, recently described in *Y. pestis* (18, 19). YbtP and YbtQ are thought to be involved in the uptake of the ferric yersiniabactin and YbtS might be involved in biosynthesis of the yersiniabactin in *Y. pestis*.

Integrase. The next ORF has an opposite transcriptional polarity to *irp6* to *irp9* (Fig. 1A). It has high similarity to the genes encoding putative integrase from *Y. pseudotuberculosis* (4) and to the P4 prophage integrase (6). In contrast to the putative integrase genes in *Y. pseudotuberculosis* and *Y. pestis* (4, 21), a TAA stop codon interrupts this ORF, henceforth designated *intB*, 415 bp downstream of the ATG start codon. The 420-amino-acid (aa) polypeptide that can be predicted from the *intB* pseudogene sequence shows 98% identity to the putative integrase of *Y. pseudotuberculosis* and 49.7% identity to the integrase of the prophage P4.

The int520 and int1597 primers amplifying the putative integrase gene were used to generate a probe for Southern hybridization with different isolates of *Y. pestis*, *Y. pseudotuberculosis* O1A, *Y. enterocolitica*, and *E. coli*. Surprisingly, two reactive bands appeared in both *Y. pseudotuberculosis* PB1 and *Y. pestis* KIM, suggesting the presence of two copies of *intB* (or, alternatively, of a sequence highly identical to the designed probe), in contrast to one copy present in *Y. enterocolitica* WA-C and *E. coli* 12860 (data not shown). The DNA of European *Y. enterocolitica* serotype O:9, O:5,27, and O:3 (BG 2, 3, and 4, respectively) strains did not hybridize with the *intB* probe. The nonpigmented *Y. pestis* KIM isolate, which had lost the 102-kb *pgm* locus, retained one band hybridizing with the *intB* probe.

intB was sequenced in several HPI-positive isolates. In contrast to *Y. enterocolitica* WA-C, a T-to-G change turned a stop codon into a GAA triplet in *Y. pestis* KIM, *Y. pseudotuberculosis* PB1, and five HPI-positive *E. coli* isolates (K49, K235, D-1041-86, C-4441, and 12860). Two other *Y. enterocolitica* 1B representatives (serotypes O:13 and O:20) contain the terminating codon in the same position as in serotype O:8 WA-C and 8081 strains.

ERIC sequence. A 127-bp enterobacterial repetitive intergenic consensus (ERIC) sequence (26), also known as the

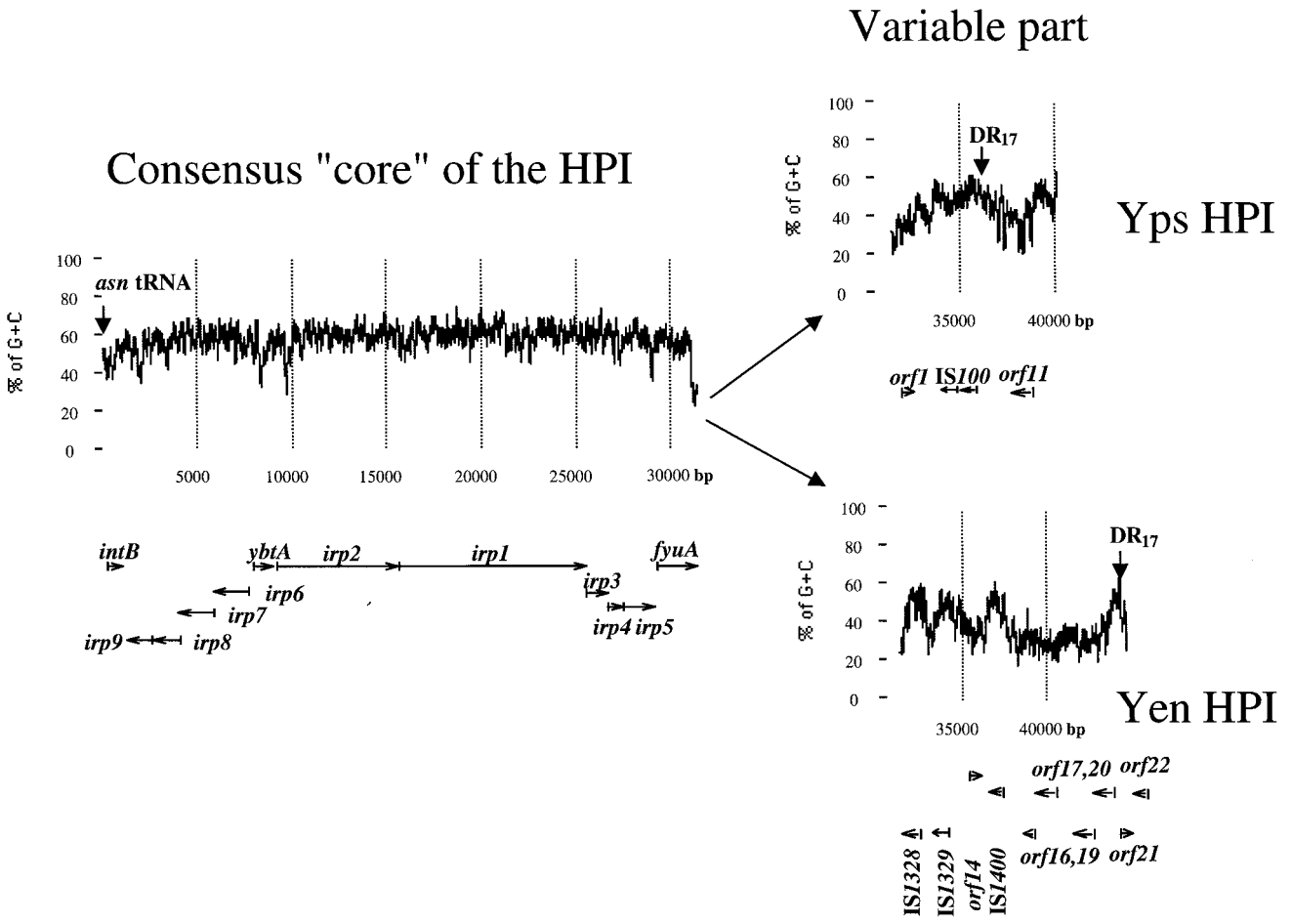

FIG. 2. Complete structure and the G+C content of the HPIs in *Y. pseudotuberculosis* PB1 and *Y. enterocolitica* WA-C. Arrows below the graph show position of genes and direction of transcription. Vertical arrows show the borders of the HPI, the left DR_{17} within *asn* tRNA defines the left border, and the right DR_{17} defines the right border.

intergenic repeated unit (IRU) (42), was recognized in the promoter of the *ybtA* yersiniabactin regulator (Fig. 3). D7 and D340 primers, designed to amplify the *ybtA* promoter, yielded a 229-bp product in *Y. pseudotuberculosis* PB1, *Y. pestis* KIM, and HPI-positive *E. coli* strains (K49, K235, D-1041-86, C-4441, and 12860). In contrast, the same primers amplified a larger (354-bp) product in all *Y. enterocolitica* 1B isolates (O:8, O:13, O:20, and O:21). Sequencing of the *Y. enterocolitica* 1B amplicons demonstrated that the 125-bp DNA insertion represents an ERIC sequence.

The ERIC element positioned within the *ybtA* promoter sequence is almost identical to the consensus ERIC sequence (26, 42). Such ERIC motifs are present in multiple copies in intergenic regions or in untranslated regions upstream or downstream of ORFs in various genomes. However, the possible function of the ERIC sequence is still enigmatic.

The *ybtA* promoter, as well as promoters of the other genes that control yersiniabactin biosynthesis and uptake, contains putative binding sites for the YbtA transcriptional regulator (17). The YbtA-binding site has a palindromic structure with inverted and direct repeats. Six nucleotides, TATACC, of the middle part of the *ybtA* operator are identical to six of seven nucleotides (TATACCC) representing the inverted repeats of the ERIC consensus (Fig. 3). This sequence seems to be a recognition site for integration of the ERIC element that possibly exploits a site-specific mechanism of integration into a target site. Taken together, these observations suggest that integration of the ERIC sequence modifies the secondary structure of the *ybtA* promoter in *Y. enterocolitica*, resulting in modulation of yersiniabactin activity (36a).

Right (3′) end of the HPI. The size of the 3′ end of the HPI differs between the evolutionary groups. We determined a 14-kb sequence downstream of the *fyuA* gene in *Y. enterocolitica* WA-C and compared it to the corresponding sequence of *Y. pseudotuberculosis* PB1.

The right end of the Yen HPI contains 13 ORFs (Fig. 1B; Table 3). Six of them are putative transposases of four insertion elements, IS*1328*, IS*1329* (two ORFs), IS*1400* (two ORFs) and ORF13, which is a truncated transposase gene of the IS*1222* sequence. The ORFs encoding IS*1328*, IS*1329*, and IS*1400* transposases are transcribed in the same orientation. Comparing the Yen and Yps HPI sequences, we determined the nucleotide sequence of an IS*3* family IS*1329* mobile element (designated RS3 by Carniel et al. [8]) and defined the precise location of the IS elements on the Yen HPI. Southern hybridization revealed that the 947-bp fragment located downstream of *fyuA* is common to all HPI-positive isolates, including *E. coli*, while sequences located downstream of this 947-bp fragment (left black bar in Fig. 4) are present only in Yen HPI. Proteins that might be encoded by the three ORFs, ORF16, ORF17, and ORF19 (Table 3), showed some similarity to the

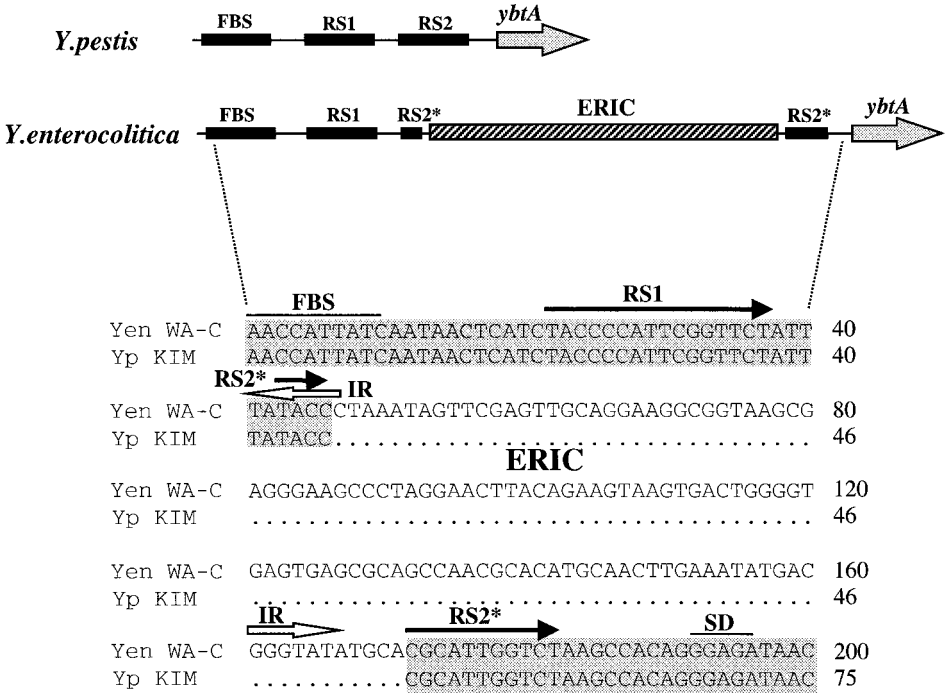

FIG. 3. Insertion of a 125-bp ERIC sequence into the *ybtA* promoter of the Yen HPI. The upper panel shows the structure of the *ybtA* promoter in *Y. pestis* and *Y. enterocolitica*. The lower panel depicts aligned nucleotide sequences of both promoters. Identical bases are boxed in grey. Black arrows show two repeated sequences (RS1 and RS2) of the *ybtA* promoter. The asterisk in the RS2* sequence depicts an interruption in the repeated sequence. Open arrows show the position and direction of the inverted repeats (IR) of the ERIC. Yen WA-C, *Y. enterocolitica* WA-C; Yp KIM, *Y. pestis* KIM; FBS, Fur protein-binding site; RS, repeated sequences, SD, potential ribosome-binding site.

hypothetical proteins YfjK (P52126) and YfjL (P52127) clustered in the *alpA-gabD* region of the *E. coli* K-12 chromosome.

ORF21 and ORF22 are located outside of the Yen HPI. The ORF21 product has 40 and 43% identity to the uracyl hydrolase of *Haemophilus influenzae* (accession no. P44782) and *E. coli* (accession no. P39219), respectively. The ORF22 product has high (86%) identity (113 of 131 aa) to a YaiL nucleotide/polynucleotide-associated enzyme of *E. coli* (accession no. U73857). Moreover, ORF22 displays 89.9% similarity to ORF9 and ORF10 located downstream of the 17-bp right direct repeat (DR_{17}) of the Yps HPI (Fig. 4; Table 4). Thus, identical chromosomal sequences, ORF22 in *Y. enterocolitica* WA-C and ORF9 and ORF10 in *Y. pseudotuberculosis* PB1, are clustered with the 3′ end of the HPI.

The right end of *Y. pseudotuberculosis* PB1 HPI contains eight ORFs, two of them encoding a putative transposase of the IS*100* element (Fig. 4; Table 4). The IS*100* insertion sequence is located 3,429 bp downstream of the *fyuA* stop codon. A 249-bp DNA fragment separates IS*100* from the DR_{17} that is identical to the 3′ end of the *asn* tRNA. ORF1 has a high

TABLE 3. ORFs defined in the right extremity of the Yen HPI and downstream of the DR_{17}

Protein	Predicted mass (kDa)	Predicted function	Amino acid identity, protein, organism (accession no.)
Right end of Yen HPI			
Tnp IS*1328*	35.5	Transposase	67% to plasmid R751 TnpA (U60777)
TnpA IS*1329*	12.9	Transposase	42% to IS*911* 12.7-kDa protein (P39213)
TnpB IS*1329*	28.7	Transposase	40% to IS*911* OrfB (AF074613)
Orf13	18.7	Transposase	98% to IS*1222* transposase (B38965)
Orf14	51.2		Unknown
TnpA IS*1400*	11.1	Transposase	88% to ORFA transposase of *Salmonella enteritidis* (Z83734)
TnpB IS*1400*	35.1	Transposase	40% to OrfB of IS*3* family (U39501)
Orf15	5.7		Unknown
Orf16	22.8		24% to YfjK of *E. coli* (P52126)
Orf17	44.5		35% to YfjK of *E. coli* (P52126)
Orf18	12		Unknown
Orf19	43.1		22% to YfjL of *E. coli* (P52127)
Orf20	43.2		24% to ComE integral protein of *Bacillus subtilis* (P39695)
Downstream of the DR_{17}			
Orf21	13.6		43% to uracyl hydrolase of *E. coli* (P39219)
Orf22	>14.7		86% to *E. coli* nucleoprotein/polynucleotide-associated enzyme (U73857)

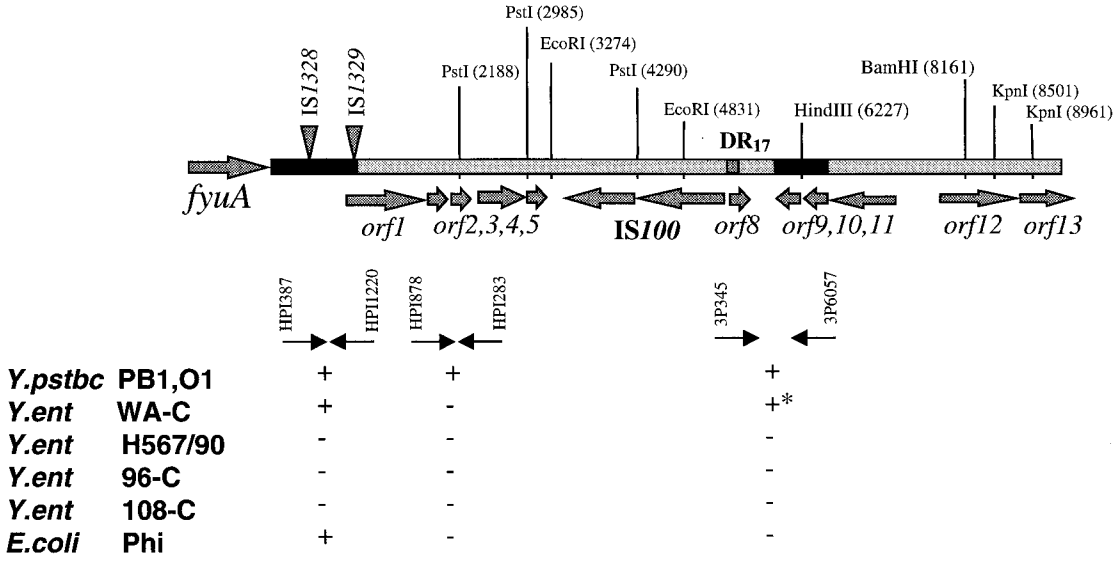

FIG. 4. Fragment of the *Y. pseudotuberculosis* PB1 chromosome with the 3′ end of the Yps HPI. The large arrow on the left indicates the position of *fyuA*. Arrows show positions of the ORFs and their direction of transcription. Black bars within the *Y. pseudotuberculosis* sequence represent regions of identity with the *Y. enterocolitica* DNA. Triangles indicate positions of the IS elements within the Yen HPI. *Pst*I, *Eco*RI, *Bam*HI, and *Kpn*I depict the positions of recognition sites of the corresponding enzymes. Small black arrows under the graph indicate PCR primers used. The DIG–11-dUTP-labelled PCR products obtained with the same primer pairs were used as hybridization probes. + and −, presence or absence, respectively, of hybridization products. +*, larger PCR amplicon in *Y. enterocolitica* WA-C; *Y.ent*, *Y. enterocolitica*; *Y.pstbc*, *Y. pseudotuberculosis*.

A+T content (63.3% A+T) and is located 874 bp downstream of the *fyuA* stop codon. It is able to encode a 20.8-kDa protein with no obvious homology to any sequence in the database besides its orthologs in the *Y. pestis* HPI (Table 4).

A small ORF, ORF2, is able to encode a 61-aa protein with 37% similarity to the hypothetical protein 88 of phage ϕR73 (G42465) and the prophage CP4-57 protein AlpA (P33997) and 36% similarity to a putative DNA-binding protein (the ORF88 product) of the bacteriophage P4 (P12552).

ORF5 may encode a 69-aa small product with possible DNA-binding activity. The ORF5 protein has 50% identity to a zinc finger region of the TraR protein of the F factor (accession no. AF005044). No function has been assigned to the TraR protein. The ORF5 product also has 37% identity to the ORF39 product of *Pseudomonas aeruginosa* phage ϕCTX (accession no. AB008550) and shows a similar level of identity to DnaK suppressor proteins of *Treponema pallidum* (33%), *H. influenzae* (31%), and *E. coli* (27%). These small proteins may play a role in interactions with DNA and may be remnants of the HPI association with self-transmissible elements. However, ORF2 and ORF5 are restricted to *Y. pestis* and *Y. pseudotuberculosis*, and are not present in the Yen HPI.

ORF9 and ORF10 are located outside the HPI and show 84 and 83% identity to a gene encoding a hypothetical nucleoprotein of *E. coli* (accession no. U73857). The ORF12 product has 75% identity to a conserved hypothetical protein of *Helicobacter pylori* (accession no. AE000549).

The ORF22 sequence of the Yen HPI (Fig. 1B) starts 839 bp downstream of the DR_{17}. It has 89.9% similarity to ORF9 and ORF10 of the Yps HPI located 356 bp downstream of the DR_{17} (Fig. 4). The same region has 98.5% similarity to the *Y. pestis* CO-92 sequence presented by the *Y. pestis* Sequencing

TABLE 4. ORFs defined in the right extremity of the Yps HPI and downstream of the DR_{17}

Protein, ORF	Predicted mass (kDa)	Predicted function	Amino acid identity, protein, organism (accession no.)
Right end of Yps HPI			
Orf1	35.4		99% to Orf73, Orf74 of *Y. pestis* HPI (AL031866)
Orf2	7.2	DNA recognition	36% to putative DNA-binding protein of phage P4 (P12552)
Orf3	6.8		100% to Orf76 of *Y. pestis* HPI (AL031866)
Orf4	18.8		100% to Orf77 of *Y. pestis* HPI (AL031866)
Orf5	8	DNA recognition	50% to TraR of F-plasmid (U01159)
TnpB IS*100*	29.2	Transposase	47% to IstB of IS*21* (P15026)
TnpA IS*100*	39.8	Transposase	32% to IstA of IS*21* (P15025)
Orf8	7.5		Unknown
Downstream of the DR_{17}			
Orf9	11		84% to *E. coli* nucleoprotein/polynucleotide-associated enzyme (U73857)
Orf10	9.1		83% to *E. coli* nucleoprotein/polynucleotide-associated enzyme (U73857)
Orf11	26.5		31% to soybean transcription factor 5 (S59539)
Orf12	34.9		75% to conserved hypothetical protein of *Helicobacter pylori* (AE000549)
Orf13	>13.2		Unknown

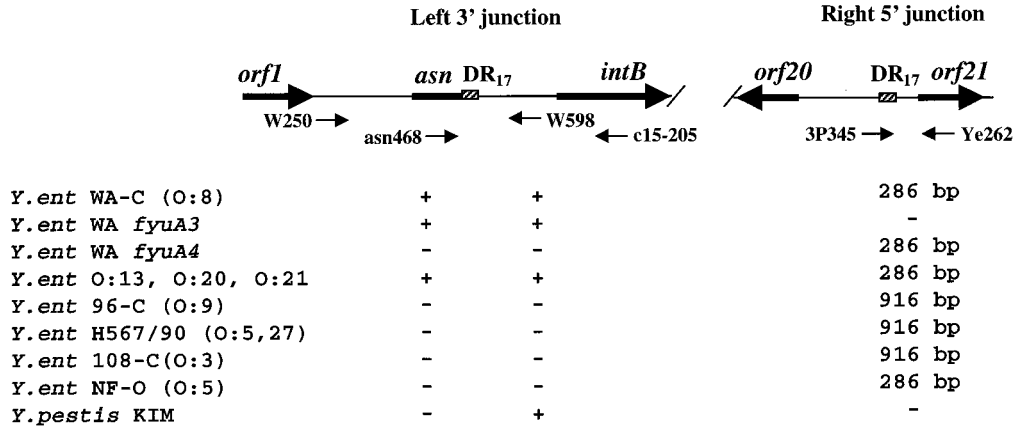

FIG. 5. Left (5′) and right (3′) junctions of the Yen HPI. Arrows in the graph show position and orientation of the genes. Black arrows under the graph show PCR primers used. The + and − indicate the presence or absence of a PCR product amplified with W250 plus W598 and asn468 plus c15-205 primer pairs. Numbers correspond to the size of the PCR products in base pairs. *Y.ent*, *Y. enterocolitica*.

Group of the Sanger Centre (39a). In contrast to *Y. pseudotuberculosis* and *Y. enterocolitica*, this DNA fragment is not contiguous with the HPI in *Y. pestis* CO-92. In *Y. pestis*, this highly conserved region is associated with another *asn* tRNA copy. Thus, the chromosomal location of the HPI is the same in *Y. enterocolitica* WA-314 and *Y. pseudotuberculosis* PB1 but differs from that in *Y. pestis*.

Junctions of the high-pathogenicity island. The 5′ *intB* junction of the HPI in *Y. enterocolitica* 8081, serotype O:8, is associated with the 3′ end of the *asnT* tRNA (8). *asnT* tRNA is also used by the HPI in *E. coli* (40). Integration of pathogenicity islands into highly conserved tRNA copies is a common feature of these islands (20). Four identical copies of the *asn* tRNA genes (*asnT*, *asnU*, *asnV*, and *asnW*) have been identified on the chromosome of *E. coli* K-12 (*E. coli* MG1655 Genome Project; accession no. AE000289, AE000290, and AE000291) and three have been identified in *Yersinia* (4). The sequence upstream of the *asnT* tRNA gene in *Y. enterocolitica* WA-C O:8 has 84% identity to the sequences upstream of the HPI in *Y. pseudotuberculosis* O:1 (accession no. AJ009592 [4]). This agrees with the proposal that the Yen HPI in *Y. enterocolitica* WA-C and the Yps HPI in *Y. pseudotuberculosis* PB1 occupy the same *asn* tRNA copy.

To test whether the HPI is associated with the same *asnT* tRNA copy in other HPI-positive *Yersinia* strains, we performed PCR with W250 and W598 primers complementary to the sequences flanking the left junction of the HPI (Fig. 5). *Y. enterocolitica* BG 1B strains of serotypes O:8, O:13, O:20, and O:21 were positive in this PCR. *Y. pestis* KIM and *Y. enterocolitica* Ye H567/90 (O:5,27, BG3), Ye 96-C (O:9, BG2), Ye 108-C (O:3, BG4), and NF-O (O:5, BG 1A) were negative in PCR with these primers. In contrast, PCR with the asn468 (annealing to the conserved part of *asn* tRNA) and c15-205 (annealing to the middle of *intB*) primers resulted in a PCR product of the same size in all HPI-positive isolates. This indicates that the HPI is integrated into the same *asnT* gene in all four serotypes of *Y. enterocolitica* BG 1B.

A 22-bp stretch of identical DNA, gtCCAGTCAGAGGAG CCAAaTT, can be recognized in the 3′ *fyuA* junction of both HPIs. This short fragment is a 20-bp imperfect duplication (or a DR_{17}) of the 3′ end of the *asn* tRNA. Such short duplications are hallmarks of site-specific recombination, whereas the 3′ end of tRNA serves as the core of the bacterial attachment site (*att*) for integration of temperate phages and conjugative plasmids (7, 38).

The HPI *att* site is unoccupied in *Y. enterocolitica* biogroup 1A. Primers W250 (located upstream of the *asnT* tRNA) and Ye262 (anneals downstream of DR_{17}) flank the HPI (Fig. 5). As expected, they failed to amplify the whole HPI in *Y. enterocolitica* WA-C and 8081. However, these primers successfully amplified a 515-bp fragment in avirulent *Y. enterocolitica* NF-O (O:5, BG 1A). The sequence of this amplicon contains a 16-bp DNA stretch identical to DR_{17} and overlaps with the sequences flanking the junctions of the HPI in *Y. enterocolitica* WA-C (Fig. 6).

Y. enterocolitica of other biogroups such as Ye108-C (O:3, BG 4), H597/90 (O:5,27, BG 3), and Ye96-C (O:9, BG 2) were negative in a PCR with the W250/Ye262 primers flanking the HPI. However, these yersiniae yielded a 916-bp PCR product with primers 3P345 (overlaps with the DR_{17}) and Ye262 (Fig. 5). This amplicon was 630 bp larger than the product amplified with the same primers in *Y. enterocolitica* WA-C. The PCR product reveals identity over 90 bp to the sequence located 134 bp downstream of the right HPI junction in yersiniae lethal for mice. Negative results with the HPI-flanking primers and presence of an additional DNA fragment adjacent to the HPI *att* site imply that the same recognition site can be used for integration of different DNA in American (BG 1B) and European (BG 2, 3, and 4) *Y. enterocolitica*. In turn, the *att* site (*asnT* RNA) that is used by the Yen HPI in *Y. enterocolitica* BG 1B strains is unoccupied in avirulent *Y. enterocolitica* O:5 BG 1A isolates.

Deletions in *Y. enterocolitica* HPI are imprecise. The yersiniabactin receptor FyuA has a dual function, since it also serves as a receptor for the *Y. pestis* bacteriocin pesticin (31, 33). Thus, pesticin sensitivity can be used as a selective marker for the presence of FyuA. Previously we selected several *Y. enterocolitica* pesticin-resistant spontaneous mutants that failed to produce the yersiniabactin on CAS agar (35). This is indicative that yersiniabactin biosynthetic genes were coinactivated with the yersiniabactin receptor gene. The WA *fyuA3* and WA *fyuA4* mutants were resistant to the pesticin due to deletions in *fyuA*. We have analyzed both mutants for the presence of the HPI genes (Table 5). *intB* through *irp4* sequences were detected in the WA *fyuA3* mutant but were absent from the WA *fyuA4* mutant. Both mutants lost IS*1328* and IS*1329* insertion

```
5′end     GTAGAACGGCGGACCGTTAATCCGTATGTCACTGGTTCGA  486
NF-O      GTAGAACGGCGGACTGTTAATCCGTATGTCACTGGTTCGA  183
3′end     ........................................    0

5′end     GTCCAGTCAGAGGAGCCAATTTTCTGTTTTCATA        520
NF-O      GTCCAGTCAGAGGAGCCATATTAGAGAAGCCCGCTTAAGG  223
3′end     TCCCAGTCAGAGGAGCCAAATTAGAGAAACCCGCTT.AGG   39

5′end
NF-O      AAACTTAAGCGGGCTTTTTGCTTTACTCATAACCTGATAT  263
3′end     CAAC.TAAGCGGGTTTTTTGCTTTACTCATAGCCTGATAT   78
```

FIG. 6. Unoccupied attachment site of the HPI in *Y. enterocolitica* NF-O. The NF-O sequence was amplified with primers W250 and Ye262 (Fig. 5) and aligned with the 5′ and 3′ boundaries of the Yen HPI. Grey boxes show identical nucleotide sequences and a 16-bp part of the DR_{17}. Numbers on the right show relative positions of nucleotides in base pairs. 5′ end, 5′ boundary of the Yen HPI, *asnT* RNA; 3′ end, 3′ boundary of the Yen HPI; NF-O, a PCR product amplified by the W250 and Ye262 primers in *Y. enterocolitica* NF-O.

sequences and either of the junctions (Fig. 5). However, WA *fyuA4* contains the IS*1400* element as well as the right junction of the HPI. WA *fyuA3* and WA *fyuA4* were negative with the W250 and Ye262 primers that amplified the island-free attachment site in *Y. enterocolitica* NF-O. In summary, WA *fyuA4* has lost the HPI sequences upstream of IS*1400*, whereas WA *fyuA3* has lost the right extremity of the island downstream of the *irp4* gene. Thus, spontaneous elimination of the Yen HPI, which results in the inactive yersiniabactin iron acquisition system, occurs through imprecise deletions in contrast to the precise excision described for the Yps HPI (4).

DISCUSSION

The HPI of *Yersinia* spp. is responsible for lethality for mice and for biosynthesis and uptake of the siderophore yersiniabactin. Sequence comparison depicts two evolutionary lineages of the HPI, *Y. enterocolitica* (Yen HPI) and *Y. pestis*/*Y. pseudotuberculosis* (Yps HPI), at the nucleotide level (21, 36) as well as in the genetic organization of the *fyuA* end of the island (8). We have determined the complete size of the Yen HPI to be 43,393 bp, which is 7.3 kb larger than that of the corresponding HPI in *Y. pseudotuberculosis*. Such a difference infers the presence of additional genes on the Yen HPI, which might support functions different from yersiniabactin-mediated iron acquisition.

Yersiniabactin-mediated iron acquisition is thought to be the main function of the HPI (19), and genes involved in yersiniabactin biosynthesis, transport, and regulation (*irp1* to *irp9*, *ybtA*, and *fyuA*) are clustered on the "core" of the HPI. The P4-like integrase gene *intB* resides on the 5′ end of the HPI next to the *asn* tRNA bacterial attachment site and belongs to the "core" of the island as well. The genes of the "core" show extremely high conservation in both evolutionary lineages and are characterized by a higher G+C (57.5 mol%) content than average in yersiniae (48 mol%) (2, 4, 19, 36). In contrast, the second component of the HPI is an AT-rich region that is completely different in the Yen HPI and Yps HPI. Thus, the genes of the HPI variable region are not expected to be involved in yersiniabactin production.

We could identify 13 ORFs within the *fyuA* end of the Yen HPI (Table 3). Six of them encode putative transposases of the four insertion sequences IS*1328*, IS*1329*, IS*1400*, and IS*1222*. The *fyuA* end of the Yps HPI in *Y. pseudotuberculosis* contains eight ORFs (Table 4). Two of them encode a putative transposase of IS*100*, while two other ORFs encode products with similarities to putative DNA-binding proteins. These proteins might be the remnants of self-transmissible elements, which were lost during stabilization of the HPI. The ORF2 and ORF5 products might be involved in DNA recognition, but they are not sufficient for the horizontal transfer of the island. Moreover, these proteins are not present in the *Y. enterocolitica* or *E. coli* HPI.

Yersinia might have acquired the HPI horizontally from a common progenitor with a high G+C content. The HPI has evolved divergently in the two evolutionary lineages, although the DNA similarity between the yersiniabactin genes of the two groups is about 98%. *Y. enterocolitica* 1B strains are the only ones that produce a halo on a CAS indicator agar, indicating yersiniabactin production. Actually, the yersiniabactin is the sole endogenous siderophore of *Y. enterocolitica*. *Y. pestis*, although containing a complete set of yersiniabactin genes, appears to be CAS negative. The fine-tuning of yersiniabactin genes to specific requirements of the *Y. enterocolitica* cell might be achieved in a stepwise mode by a single integration of the ERIC element into the promoter of the *ybtA* yersiniabactin regulator in *Y. enterocolitica*. The ERIC sequence modifies the structure of the *ybtA* operator and thus might be responsible for the different expression of the yersiniabactin biosynthetic genes in yersiniae (36a).

The HPI is associated with the *asn* tRNA genes in *Y. enterocolitica* (8), *Y. pseudotuberculosis* (4), *Y. pestis* (21), and *E. coli* (40). The Yps HPI can be inserted into any of the three *asn* tRNA copies of *Y. pseudotuberculosis* (4) and can use different *asn* tRNA genes in *Y. pestis* and *Y. pseudotuberculosis* (21). The comparison of the HPI integration sites in two *Y. pseudotuberculosis* strains, described in recent publications (4, 21), dem-

TABLE 5. Presence of the HPI-associated sequences in *Y. enterocolitica* strains

Strain	Presence of:									
	attL[a]	*intB*	*irp2*	*irp1*	*irp4*[a]	*fyuA*	IS*1328*	IS*1329*	IS*1400*	*attR*[a]
WA-C	+	+	+	+	+	+	+	+	+	+
WA *fyuA3*	+	+	+	+	+	−	−	−	−	−
WA *fyuA4*	−	−	−	−	−	−	−	−	+	+

[a] The presence of these sequences was checked by a PCR. The presence of the other sequences was proved by Southern hybridization.

onstrates that the Yps HPI integrates into two different *asn* tRNA copies in these strains. In contrast to the Yps HPI, the Yen HPI is stably integrated into the same *asnT* RNA gene in all serotypes of *Y. enterocolitica* BG 1B strains. Therefore, the HPI seems to be "immobile" in *Y. enterocolitica*, perhaps due to the inactivated putative integrase. *Y. enterocolitica* is thus not expected to be the original donor of the HPI that is widely distributed among the members of the *Enterobacteriaceae* (40). The presence of the *Y. pestis*-type HPI in the *Enterobacteriaceae* supports this prediction.

The Yen HPI, like the Yps HPI, is flanked by a 17-bp perfect duplication of the 3′ end of the *asn* tRNA gene. Such duplications indicate a site-specific recombination event that results in integration of prophages and plasmids (7). The excision of the integrated units is predominantly precise and leads to the reconstruction of the original attachment site. A precise excision seems to be responsible for the HPI disintegration in *Y. pseudotuberculosis* O:1A (4). In contrast, deletions of the Yen HPI sequences in two pesticin-resistant mutants resulted in different endpoints and extensions. Moreover, the direct repeats of the island do not play a role in these deletion events. Three complete IS elements, which are present in the Yen HPI, might be responsible for the above deletions. Consequently, in contrast to *Y. pseudotuberculosis*, the precise excision of the Yen HPI is a rare or perhaps even impossible event in *Y. enterocolitica*.

We have identified an "island-free" bacterial *att* site for the HPI in apathogenic *Y. enterocolitica* NF-O (serotype O:5, BG 1A) (Fig. 6). The same site is "occupied" in HPI-negative yersiniae of BG 2, 3, and 4. This indicates that the *asn* tRNA genes might be used as integration sites for a foreign DNA in human pathogenic yersiniae.

The widespread presence of the HPI in *E. coli* of different pathotypes (40) implies an efficient mechanism of its transfer. Temperate phages or transmissible plasmids are candidates as HPI vehicles. The presence of a variable AT-rich "additive" to the highly conserved "core" points to a passive HPI transfer by a head-full phage transduction. It is also possible that the mobility genes were already lost by the island as in the locus of enterocyte effacing (LEE) island in enteropathogenic *E. coli* strains compared to enterohemorrhagic *E. coli* O157:H7 (30). Alternatively, the LEE island in O157:H7 may acquire the mobility genes. ORF2 and ORF5 with possible DNA-binding ability might be remnants of a mobility fraction of the ancestral HPI.

Different pathotypes of *E. coli* carry the yersiniabactin "core" of the HPI (40). In addition to the yersiniabactin iron acquisition system, *E. coli* has the enterochelin system, with higher affinity for iron (10). Reportedly, isolated *irp2*-positive *Y. pseudotuberculosis* O:3 strains do not express siderophore activity on the CAS agar and lack the yersiniabactin receptor (4, 36). Therefore, one can envisage alternative functions of the HPI-encoded genes besides production of the yersiniabactin and iron uptake. Modulation of cellular host defense (1, 13) may be a complementary function of the yersiniabactin. Determination of alternative functions and mechanisms of HPI transfer will provide interesting insights into the evolution of genomic islands.

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