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Evidence for Two Evolutionary Lineages of Highly Pathogenic *Yersinia* Species

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Sensitivity to *Yersinia pestis* bacteriocin pesticin correlates with the existence of two groups of human pathogenic yersiniae, mouse lethal and mouse nonlethal. The presence of the outer membrane pesticin receptor (FyuA) in mouse-lethal yersiniae is a prerequisite for pesticin sensitivity. Genes that code for FyuA (*fyuA*) were identified and sequenced from pesticin-sensitive bacteria, including *Y. enterocolitica* biotype 1B (serotypes O8, O13, O20, and O21), *Y. pseudotuberculosis* serotype O1, *Y. pestis*, two known pesticin-sensitive *Escherichia coli* isolates (*E. coli* Phi and *E. coli* CA42), and two newly discovered pesticin-sensitive isolates, *E. coli* K49 and K235. A 2,318-bp *fyuA* sequence was shown to be highly conserved in all pesticin-sensitive bacteria, including *E. coli* strains (DNA sequence homology was 98.5 to 99.9%). The same degree of DNA homology (97.8 to 100%) was established for the sequenced 276-bp fragment of the *irp2* gene that encodes high-molecular-weight protein 2, which is also thought to be involved in the expression of virulence by *Yersinia* species. Highly conserved *irp2* was also found in all pesticin-sensitive *E. coli* strains. On the basis of the *fyuA* and *irp2* sequence homologies, two evolutionary groups of highly pathogenic *Yersinia* species can be established. One group includes *Y. enterocolitica* biotype 1B strains, while the second includes *Y. pestis*, *Y. pseudotuberculosis* serotype O1, and *irp2*-positive *Y. pseudotuberculosis* serotype O3 strains. *E. coli* Phi, CA42, K49, and K235 belong to the second group. The possible proximity of these two iron-regulated genes (*fyuA* and *irp2*), as well as their high levels of sequence conservation and similar G+C contents (56.2 and 59.8 mol%), leads to the assumption that these two genes may represent part of an unstable pathogenicity island that has been acquired by pesticin-sensitive bacteria as a result of a horizontal transfer.

Human pathogenic yersiniae are known either as the causative agent of plague (*Yersinia pestis*) or as food-borne pathogens (*Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) that cause intestinal diseases (5). The pathogenicity of each of these species depends on the presence of a closely related 70-kb virulence plasmid, pYV (1, 17, 22, 38). Although the presence of pYV is absolutely required for pathogenicity, strains of these species can be distinguished by their virulence for mice, suggesting that the mouse virulence or lethality trait is determined by an additional chromosomal locus. Accordingly, pYV-harboring yersiniae can be divided into two groups. (i) *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* biotype 1B belong to the mouse-lethal group (50% lethal intravenous dose [LD_{50}], $<10^3$ organisms), and (ii) the mouse-nonlethal group (intravenous LD_{50}, $>10^5$ organisms) consists of *Y. enterocolitica* strains of non-1B biotypes (8, 11, 28).

It has been shown that the mouse lethality trait in yersiniae is closely related to sensitivity to the lethal effect of pesticin, a bacteriocin produced by *Y. pestis* (6, 7, 26). The exceptions to this rule are *Y. pestis* strains which are immune to pesticin activity because of the presence of the immunity gene (31, 46) and non-serotype O1 strains of *Y. pseudotuberculosis* which are insensitive to pesticin (6, 7, 29, 45).

On the other hand, some rare representatives of *Escherichia coli* (the colicin indicator strain *E. coli* Phi and colicin E2 producer strain *E. coli* CA42) are known to be sensitive to pesticin, but they do not exhibit high-level mouse virulence (6, 18, 45).

Recently, we demonstrated that pesticin sensitivity, at least in *Y. enterocolitica* WA-314 (serotype O8), is mediated by a 71-kDa iron-repressible TonB-dependent outer membrane protein (29, 39). The pesticin receptor was designated FyuA because it also functions as a receptor for ferric yersiniabactin uptake (29). Yersiniabactin is a siderophore which is produced by *Y. enterocolitica* biotype 1B strains (24, 27, 29). We were able to demonstrate that both production of yersiniabactin and ferric yersiniabactin uptake via FyuA are required for mouse virulence (27, 29, 39). Thus, mouse virulence in yersiniae, at least in *Y. enterocolitica* biotype 1B strains, appears to be closely related to this high-affinity iron uptake system.

Recently, several groups have reported spontaneous deletions of chromosomal determinants that encode iron-repressible proteins (IRPs) (e.g., Irp B-E and high-molecular-weight proteins 1 and 2 [HMWP1 and HMWP2]) and/or pesticin sensitivity (9, 19, 20, 38, 43, 49). The loss of these determinants was associated with a reduction in virulence to mice. From these results, one might conclude that at least some of the IRPs that are associated with virulence are encoded by such an unstable 33- to 102-kb chromosomal fragment. The established nucleotide sequences of *Y. enterocolitica fyuA* (39) and *irp2* (encoding HMWP2) (23) genes prompted us to compare these sequences with sequences obtained from other pesticin-sensitive *Yersinia* and *E. coli* strains in order to elucidate a putative evolutionary relationship between these virulence traits.

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used are listed in Table 1. *Y. pseudotuberculosis* PB1 and *Y. pestis* KIM-C and EV76 were from R. R. Brubaker, East Lansing, Mich.; *Y. pestis* 6/69, devoid of the Lcr virulence plasmid, was obtained from E. Carniel, Paris, France; *Y. pseudotuberculosis* serotype O3 strains were from S. Aleksic, Hamburg, Germany; *Y. pseudotuberculosis* YP252 (serotype O1A) and YP2613 (serotype O1B) were obtained from S. Korovkin, Saratov, Russia; *Y. pseudotuberculosis* YP68 (serotype O5A) was from I. Semenova, St. Petersburg, Russia; *Y. pseudotuberculosis* K86 (serotype

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TABLE 1. Sensitivities to pesticin and presence of the *fyuA* and *irp2* genes in *Yersinia* and *E. coli* strains

Strain and serotype	Sensitivity to pesticin[a]	Presence of:		
		FyuA[b]	*fyuA*[c]	*irp2*[c]
Y. enterocolitica WA-314, O8	S	+	+	+
Y. enterocolitica WA-3, O8	R	−	−	+
Y. enterocolitica 8081, O8	S	+	+	+
Y. enterocolitica 1209-79, O13	S	+	+	+
Y. enterocolitica 1223-75-1, O20	S	+	+	+
Y. enterocolitica YE737, O21	S	+	+	+
Y. pseudotuberculosis PB1, O1	S	+	+	+
Y. pseudotuberculosis YP252, O1A	S	+	+	+
Y. pseudotuberculosis YP2613, O1B	S	+	+	+
Y. pseudotuberculosis Erlangen, O2	R	−	−	−
Y. pseudotuberculosis YP146, O3	R	−	−	−
Y. pseudotuberculosis YP200, O3	R	−	−	+
Y. pseudotuberculosis YP201, O3	R	−	−	−
Y. pseudotuberculosis YP307, O3	R	−	−	+
Y. pseudotuberculosis YP346, O3	R	−	−	+
Y. pseudotuberculosis YP714, O3	R	−	−	−
Y. pseudotuberculosis YP1134, O3	R	−	−	+
Y. pseudotuberculosis YPIII, O3	R	−	−	−
Y. pseudotuberculosis YP3813, O4A	R	−	−	−
Y. pseudotuberculosis K86, O4B	R	−	−	−
Y. pseudotuberculosis 84-99, O4B	R	−	−	−
Y. pseudotuberculosis YP68, O5A	R	−	−	−
Y. pestis 6/69	I	+	+	+
Y. pestis KIM-C	I/R	−	−	−
Y. pestis EV76	I/R	−	−	−
C. freundii CA31(ColA)	R	−	−	−
E. coli Ag097(ColB)	R	−	−	−
E. coli CA23(ColD)	R	−	−	−
E. coli 20741(ColE2)	R	−	−	−
E. coli CA42(ColE2)	S	+	+	+
E. coli 20743(ColE3)	R	−	−	−
E. coli CA46(ColG)	R	−	−	−
E. coli CA53(ColIa)	R	−	−	−
E. coli CA38(ColJ, ColE3)	R	−	−	−
E. coli K235(ColK)	S	+	+	+
E. coli K49(ColK)	S	+	+	+
E. coli CA7(ColM, ColV)	R	−	−	−
E. coli K311(ColV)	R	−	−	−
E. coli Phi	S	+	+	+
E. coli K-12 DH5α	R	−	−	−
E. coli 117/86, O6:H$^-$	R	−	−	−
E. coli ED142, O111	R	−	−	−
E. coli 12810, O114:H2	R	−	−	−
E. coli 3715/67, O127	R	−	−	−
E. coli 147/1, O128:H$^-$	R	−	−	−
E. coli 76-5, O143	R	−	−	−
E. coli 4218/93, O157:H7	R	−	−	−

[a] S, sensitive; I, immune; R, resistant.

[b] The presence of the FyuA receptor was analyzed by Western blotting with anti-FyuA polyclonal antibodies.

[c] The presence of the *fyuA* and *irp2* genes was proved by Southern blotting with digoxigenin-labelled, PCR-generated *fyuA* and *irp2* gene probes, respectively.

O4B) and 84-99 (serotype O4B) were provided by H. Fukushima, Shimane, Japan. The *Y. enterocolitica* strains were from the collection of the Institute for Hygiene and Microbiology, Würzburg, Germany (27, 29). Colicin-producing strains were obtained from G. Schmidt, Borstel, Germany, and V. Braun, Tübingen, Germany. Clinical enteric *E. coli* isolates of different pathotypes (*E. coli* 117/86, O6:H$^-$ [ETEC]; *E. coli* 147/1, O128:H$^-$ [ETEC]; *E. coli* ED142, O111 [EHEC]; *E. coli* 4218/93, O157:H7 [EHEC]; *E. coli* 12810, O114:H2 [EPEC]; *E. coli* 3715/67, O127 [EPEC]; and *E. coli* 76-5, O143 [EIEC]) were provided by H. Karch, Würzburg, Germany. *Y. enterocolitica* WA-3 was isolated as a spontaneous pesticin-resistant mutant derived from WA-314 which had acquired a deletion of the *fyuA* gene. Strains were grown in Luria broth or on Luria-Bertani agar plates (Difco Laboratories, Detroit, Mich.) at 28 (*Yersinia* spp.) or 37°C (*E. coli*). Pesticin assays, isolation of outer membrane proteins, antiserum preparation, and Western immunoblotting were performed as previously described (29).

DNA manipulations. Chromosomal DNAs from various bacterial species were isolated according to the method of Davis et al. (13) and quantified by UV-VIS spectroscopy, with absorption measured at 260 nm. Southern blot hybridizations were performed with digoxigenin-labelled PCR probes by using primers P161 and P191 (see Fig. 1) for the *fyuA* gene and primers P242 and P505 for the *irp2* gene and DIG-11-dUTP according to the Boehringer Mannheim Biochemica protocol.

Chromosomal DNA isolated from *Y. pseudotuberculosis* PB1 was partially digested with endonuclease *Sau*3AI, and 20- to 30-kb DNA fragments were ligated into the *Bam*HI site of vector pLAFR2 (21) to create a cosmid genomic library. A library was packaged in vitro and introduced into *E. coli* S17-1 (44). A total of 2,112 clones were screened for the presence of the *fyuA* gene by colony hybridization with the digoxigenin-labelled *fyuA* probe. Plasmid DNA isolated from the *fyuA*-positive clone was digested with *Eco*RI and *Sal*I-*Pst*I endonucleases and subcloned into appropriate sites of the pBluescript KSII vector (Stratagene cloning systems). Constructed recombinant plasmids that carried the *fyuA* gene were used as templates for PCR reactions to confirm the sequencing results obtained with *Y. pseudotuberculosis* PB1 chromosomal DNA.

Oligonucleotide design. Oligonucleotides that corresponded to the region from bp 241 to bp 526 of the *irp2* gene (P242, 5′-[241]AAGGATTCGCTGTT ACCGGAC-3′ and P505, 5′-[526]TCGTCGGGCAGCGTTTCTTCT-3′) were designed according to published DNA sequence information (23) and were used as amplification and sequencing primers. Oligonucleotides that had been designed to be the sequencing primers for sequencing the *Y. enterocolitica* WA-314 *fyuA* gene (39) were applied as amplification and sequencing primers for determination of the primary DNA structure of the *fyuA* genes of pesticin-sensitive bacteria (Fig. 1). All primers were designed with Oligo (version 4.0) primer analysis software (National Biosciences).

PCR conditions. PCR amplifications were performed in an automated thermal cycler (TRIO Thermoblock; Biometra) as described by Saiki et al. (40) with *Taq*I polymerase (15) and different pairs of oligonucleotide sequencing primers that had been obtained from Roth (Karlsruhe, Germany). The initial denaturation step (94°C, 5 min) was followed by 30 cycles of denaturation, annealing, and extension with one final extension step. The temperatures and times for the last two steps varied according to the primers utilized. Five microliters from each PCR were electrophoresed on 1% agarose gels (41). PCR amplification products were purified and concentrated with JET PURE beads (GENOMED GmbH, Bad Oeynhausen, Germany) according to the protocol of the manufacturer. In certain cases, PCR amplification products were also gel purified by using a Geneclean kit (Diagen GmbH, Hilden, Germany).

DNA sequencing and sequence comparison. DNA sequencing was performed by the chain terminating method with a model 373A DNA sequencer (Applied Biosystems, Inc.). The obtained DNA sequences were aligned and analyzed with the HIBIO Mac DNASIS program (Hitachi Software Engineering Co.) and with the programs included in the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this study were submitted to the EMBL data bank under the following accession numbers: Z35104, *Y. pestis* 6/69 *fyuA*; Z35105, *E. coli* CA42 *fyuA*; Z35106, *E. coli* Phi *fyuA*; Z35107, *Y. pseudotuberculosis* PB1 *fyuA*; Z35485, *Y. enterocolitica* 1209-79 *fyuA*; Z35486, *Y. enterocolitica* 8081 *fyuA*; Z35487, *Y. enterocolitica* 1223-75-1 *fyuA*; Z35496, *Y. enterocolitica* YE737 *fyuA*; Z38064, *E. coli* K235 *fyuA*; Z38065, *E. coli* K49 *fyuA*; Z35446, *Y. enterocolitica* 1209-79 *irp2*; Z35447, *Y. enterocolitica* 1223-75-1 *irp2*; Z35448, *Y. enterocolitica* YE737 *irp2*; Z35449, *Y. pestis* 6/69 *irp2*; Z35450, *Y. enterocolitica* WA-314 *irp2*; Z35451, *Y. pseudotuberculosis* YP346 *irp2*; Z35452, *E. coli* Phi *irp2*; Z35453, *Y. pseudotuberculosis* YP307 *irp2*; Z35454, *Y. enterocolitica* 8081 *irp2*; Z35455, *Y. pseudotuberculosis* PB1 *irp2*; Z35456, *E. coli* CA42 *irp2*; Z35457, *Y. pseudotuberculosis* YP200 *irp2*; Z35477, *Y. pseudotuberculosis* YP1134 *irp2*; Z35488, *E. coli* K49 *irp2*; Z46919, *E. coli* K235 *irp2.*

RESULTS

Presence of homologous *fyuA* sequences in pesticin-sensitive microorganisms. It has been shown that the presence of the FyuA receptor is a prerequisite for pesticin sensitivity, as well as for the ability to utilize the yersinia siderophore yersiniabactin in *Y. enterocolitica* WA-314 (serotype O8) (24, 29, 39). As expected, *Y. enterocolitica* serotype O8, O13, O20, and O21 strains (all biotype 1B strains) and *Y. pseudotuberculosis* serotype O1, O1A, and O1B strains were sensitive to pesticin, while *Y. pseudotuberculosis* serotype O2, O3, O4A, O4B, and O5A strains were insensitive to pesticin action (Table 1). To determine the mechanism of *Y. pseudotuberculosis* serotype O2, O3, O4A, O4B, and O5A insensitivity to pesticin, Southern blotting with a *fyuA* gene probe and Western blotting with rabbit anti-FyuA serum were performed. According to the results obtained, the *fyuA* gene and FyuA receptor were absent in *Y.*

FIG. 1. Nucleotide sequence of the *E. coli* Phi *fyuA* gene. Nucleotides are numbered, beginning with the ATG start codon. Boldfaced letters show the positions of nucleotides found to be mutated in the analyzed *fyuA* sequences. −35, promoter sequence; sd, Shine-Dalgarno ribosome-binding site; T, terminator stem-loop structure. Start (ATG) and stop (TGA) codons are indicated by asterisks. Fur-box, Fur protein-binding site (also contains −10 promoter sequence TATCAT [bp −65]). Oligonucleotide sequences used as primers (P) are underlined, and a plus or minus sign indicates the orientation (5′ to 3′) of each primer; several oligonucleotide sequences were used to create primers in both orientations (e.g., P176 and P15, P171 and P160, etc.). This sequence was deposited in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under accession number Z35106.

pseudotuberculosis serotype O2, O3, O4A, O4B, and O5A strains and present in all pesticin-sensitive bacteria, including *E. coli* Phi and *E. coli* CA42 (Table 1). From this, we conclude that *Y. pseudotuberculosis* serotype O2, O3, O4A, O4B, and O5A strains are resistant to pesticin because they lack the *fyuA* gene and the pesticin receptor. Thus, sensitivity to pesticin is manifest only in the presence of FyuA.

Y. pestis KIM-C and EV76 were immune to pesticin action because of the presence of the pesticin immunity gene on a 9.5-kb plasmid (46). Nevertheless, these strains also lacked the *fyuA* gene, possibly as the result of a deletion (19, 20) that may have taken place in these two strains during subculture on artificial media. Thus, these *Y. pestis* strains had become resistant to pesticin. In contrast, *Y. pestis* 6/69 was both *fyuA* positive and FyuA positive, but it was pesticin immune because of the presence of the pesticin immunity gene on a 9.5-kb plasmid.

We have analyzed different colicin-producing *E. coli* strains in addition to a colicin A-producing *Citrobacter freundii* strain and seven clinical enteric *E. coli* isolates of different pathotypes (EHEC, EIEC, EPEC, and ETEC). *E. coli* K-12 DH5α was used as an established negative control for the presence of the *fyuA* gene (39). Two *E. coli* strains that produce colicin K were found to be pesticin sensitive (Table 1). However, neither this colicin nor colicin E2, produced by strain *E. coli* CA42, was active against *Y. enterocolitica* serotype O8 strains, indicating that these colicins use receptors other than FyuA. *E. coli* K49 and K235 were FyuA positive by Western immunoblotting with anti-FyuA antiserum (Fig. 2) and possessed *fyuA* genes according to Southern hybridizations of their chromosomal DNAs with the *fyuA* gene probe (Fig. 3). The band that hybridized with the *fyuA* probe was the same size for both *Y. enterocolitica* and *E. coli* pesticin-sensitive strains, while the fragment that hybridized with the *irp2* probe was one of higher molecular weight in all four *E. coli* strains (Fig. 3). This is due to loss of the internal *Eco*RI restriction site that is present in all *Y. enterocolitica* biotype 1B strains and absent from other *irp2*-positive bacteria (data not shown). All four pesticin-sensitive *E. coli* strains were identified by the API 20E system as *E. coli* strains with more than 98% probability. These results prove that the *fyuA* gene is more widely distributed than previously assumed.

***fyuA* is highly conserved among pesticin-sensitive microorganisms.** PCR oligonucleotide primers derived from the *fyuA* sequence of *Y. enterocolitica* WA-314 (serotype O8) were designed to obtain PCR-amplified *fyuA* sequences from other pesticin-sensitive microorganisms (Fig. 1). PCR-amplified products of the expected size were obtained only for pesticin-sensitive *Yersinia* and *E. coli* strains. The amplified *fyuA* se-

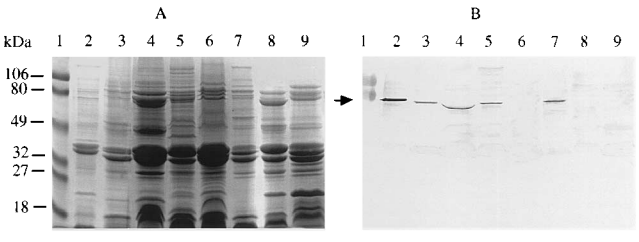

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Coomassie brilliant blue stain) of outer membrane proteins of *Y. enterocolitica* WA-314 and *E. coli* strains (A) and corresponding Western immunoblot developed with anti-FyuA (B). Lanes: 1, marker proteins (in kilodaltons); 2, *Y. enterocolitica* WA-314; 3, *E. coli* Phi; 4, *E. coli* CA42; 5, *E. coli* K49; 6, *E. coli* DH5α; 7, *E. coli* K235; 8, *E. coli* 3715/67; 9, *E. coli* ED142. The arrow indicates the position of FyuA.

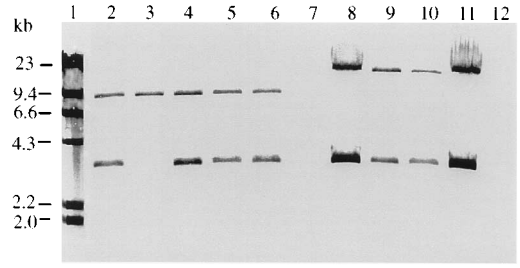

FIG. 3. Southern hybridizations of chromosomal DNAs isolated from *Yersinia* and *E. coli* strains. Chromosomal DNAs were digested to completion with *Eco*RI, and the resulting fragments were separated on 1.0% agarose gels prior to Southern blotting. Hybridizations were subsequently performed with two digoxigenin-labelled, PCR-generated probes, with primers P161 and P191 for the *fyuA* gene and primers P242 and P505 for the *irp2* gene. Lanes: 1, lambda DNA digested with *Hin*dIII (used as a molecular weight marker); 2, *Y. enterocolitica* WA-314 (serotype O8); 3, *Y. enterocolitica* WA-3, with a deleted *fyuA* gene; 4, *Y. enterocolitica* 1209-79 (serotype O13); 5, *Y. enterocolitica* 1223-75-1 (serotype O20); 6, *Y. enterocolitica* YE737 (serotype O21); 7, *E. coli* K-12 DH5α; 8, *E. coli* Phi; 9, *E. coli* CA42; 10, *E. coli* K49; 11, *E. coli* K235; 12, *Y. pseudotuberculosis* YPIII (serotype O3). The upper fragment is the result of hybridization with the *irp2* probe, and the lower one is the result of hybridization with the *fyuA* probe.

quences were sequenced and aligned, and the detected nucleotide substitutions (Fig. 1) were analyzed.

The analysis showed extremely high conservation of the *fyuA* primary DNA structure in all analyzed pesticin-sensitive bacteria (Table 2). The homologies of *fyuA* genes from different pesticin-sensitive bacteria ranged from 98.5 to 99.9%. The G+C content of the *fyuA* gene was 56.2 mol%, which is higher than the overall G+C content of the *Yersinia* chromosome (46 to 50 mol%) (2).

The positions of nucleotide substitutions (Fig. 1) and their possible effects on predicted protein structure (12) are depicted in Table 3. Most substitutions, even those in first and second codon positions, appeared to be silent.

Nevertheless, three nucleotide substitutions at positions that correspond to bp 98, 1887, and 1900 of *Y. enterocolitica fyuA* DNA influenced the FyuA structure in regions that possibly interact with the TonB protein. These regions were the amino-terminal membrane-spanning amphipathic β-sheet, the so-called TonB box, which is involved in direct interaction between the TonB protein and the receptor (30, 42), and membrane-spanning β-sheet carboxy terminus, which is well-conserved among TonB-dependent receptors (48).

Evidence for two evolutionary lineages of *fyuA* genes. Although sequences from different pesticin-sensitive bacteria are highly conserved, local differences in these 2,318-bp *fyuA* sequences (Fig. 1), namely, nucleotide substitutions (mainly transitions), insertion of a thymine nucleotide in the noncoding region of *fyuA* (bp −33) in *E. coli* CA42 and K235, and one 5-bp inversion exchange with the second DNA strand in the loop region of the terminator stem-loop structure in *E. coli* Phi, exist. It turns out that *Y. pseudotuberculosis* serotype O1 *fyuA* is the most highly conserved, while *Y. enterocolitica* serotype O8 (strains WA-314 and 8081), O13, O20, and O21 *fyuA* genes have the largest number of mutated nucleotides (Table 3).

As a result of *fyuA* DNA sequence comparisons, two groups of pesticin-sensitive bacteria can be established. Group I (*Y. enterocolitica* type) includes all biogroup 1B *Y. enterocolitica* strains (serotypes O8, O13, O20, and O21), while group II (*Y. pseudotuberculosis* type) includes *Y. pseudotuberculosis* serotype O1 strains, *Y. pestis*, and *E. coli* Phi, CA42, K49, and K235.

Genes for HMWP 2 are also highly conserved in highly pathogenic *Yersinia* and *E. coli* strains. From the results of Fetherston et al. (19, 20), one would expect the *Y. pestis* genes that encode IRPs, HMWP1, and HMWP2, as well as the pesticin receptor, to be located on a 102-kb fragment that is flanked by IS*100* sequences. The loss of this fragment leads to pesticin resistance; to loss of most of the IRPs, HMWP1, and HMWP2; and to attenuation of virulence (9, 10, 19, 20). Taking this into account, we analyzed the bacteria which had been tested for pesticin sensitivity for the presence of the *irp2* gene. By PCR and Southern hybridization (Fig. 3), *irp2* was found in all pesticin-sensitive strains, as well as in four of the eight isolates of pesticin-resistant *Y. pseudotuberculosis* serotype O3 strains studied. The most striking presence was that of *irp2* sequences in all *E. coli* pesticin-sensitive isolates, Phi, CA42, K49, and K235.

In order to define the frequency of nucleic acid substitutions in the *irp2* gene and to compare it with the frequency of substitutions in *fyuA*, we analyzed a 276-bp fragment of the *irp2* sequence (bp 241 to 526) from each *irp2*-positive isolate. The sequence data alignment is presented in Fig. 4. From these data, it becomes evident that *irp2* sequences are highly conserved among pesticin-sensitive bacteria and *irp2*-positive *Y. pseudotuberculosis* serotype O3 isolates. DNA homology between *irp2* sequences from different *irp2*-positive bacteria ranged from 97.8 to 100%.

Nevertheless, local differences in DNA composition of *irp2* sequences (Fig. 4) make it possible to distinguish between two groups of *irp2*-positive bacteria, namely, *Y. enterocolitica* biotype 1B strains (group I) and the *Y. pseudotuberculosis* group (group II), which includes *Y. pseudotuberculosis* serotype O1 and O3 *irp2*-positive strains, *Y. pestis*, *E. coli* Phi, *E. coli* K49, *E. coli* K235, and *E. coli* CA42. These two *irp2* groups coincide well with the established two groups of *fyuA*-positive strains, with the addition of pesticin-resistant, *irp2*-positive *Y. pseudotuberculosis* serotype O3 strains, thus emphasizing the existence of two evolutionary groups of pesticin-sensitive, *irp2*-positive bacteria, represented by highly pathogenic *Yersinia* spp. and some *E. coli* isolates.

TABLE 2. Comparison of the nucleotide sequence homologies (over 2,340 bp) of *fyuA* genes from various pesticin-sensitive bacteria

Strain	Homology (%)					
	Y. enterocolitica WA-314 (serotype O8)	*Y. pseudotuberculosis* PB1 (serotype O1)	*Y. pestis* 6/69	*E. coli* Phi	*E. coli* CA42	*E. coli* K49
Y. pseudotuberculosis PB1 (serotype O1)	99.0					
Y. pestis 6/69	99.1	99.8				
E. coli Phi	98.5	99.5	99.3			
E. coli CA42	98.9	99.9	99.7	99.4		
E. coli K49	98.8	99.8	99.6	99.6	99.7	
E. coli K235	98.7	99.7	99.5	99.4	99.9	99.6

TABLE 3. Comparison of nucleotide and amino acid sequences of *fyuA* genes from different pesticin-sensitive bacteria

Position (bp)	Sequence				
	Y. enterocolitica WA-314[a]	*Y. pseudotuberculosis* serotype O1	*Y. pestis*	*E. coli* Phi	*E. coli* CA[b]
−129	**T**	C	T	C	C
−81	**C**	C	C	T	C
−78	**T**	T	T	T	C
98	G**A**G/Glu	T/Val	T/Val	T/Val	T/Val
156	AC**C**/Thr	T/Thr	T/Thr	T/Thr	T/Thr
192	GT**T**/Val	C/Val	C/Val	C/Val	C/Val
225	CT**T**/Pro	C/Pro	T/Pro	C/Pro	C/Pro
291	TC**G**/Ser	A/Ser	A/Ser	A/Ser	A/Ser
366	CA**G**/Gln	G/Gln	G/Gln	A/Gln	G/Gln
402	CG**T**/Arg	A/Arg	T/Arg	A/Arg	A/Arg
642	GA**T**/Asp	C/Asp	C/Asp	C/Asp	C/Asp
756	GA**C**/Asp	C/ASP	C/Asp	T/Asp	C/Asp
795	CT**C**/Leu	G/Leu	G/Leu	G/Leu	G/Leu
798	TC**A**/Ser	G/Ser	G/Ser	G/Ser	G/Ser
799, 801	**CTT**/Leu	ATC/Ile	ATC/Ile	ATC/Ile	ATC/Ile
960	AT**G**/Met	G/Met	G/Met	A/Ile	G/Met
999	GC**C**/Ala	C/Ala	C/Ala	C/Ala	T/Ala
1084	**A**AC/Asn	G/Asp	G/Asp	G/Asp	G/Asp
1329	GA**C**/Asp	T/Asp	T/Asp	T/Asp	T/Asp
1351	**A**TA/Ile	G/Val	G/Val	G/Val	G/Val
1356	GC**T**/Ala	C/Ala	C/Ala	C/Ala	C/Ala
1410	GC**T**/Ala	C/Ala	C/Ala	C/Ala	C/Ala
1451	G**G**C/Gly	G/Gly	A/Asp	G/Gly	G/Gly
1602	AA**A**/Lys	G/Lys	G/Lys	G/Lys	G/Lys
1689	GG**C**/Gly	T/Gly	C/Gly	T/Gly	T/Gly
1848	AC**T**/Thr	C/Thr	C/Thr	C/Thr	C/Thr
1887	AT**A**/Ile	G/Met	G/Met	G/Met	G/Met
1900	**C**AT/His	T/Tyr	T/Tyr	T/Tyr	T/Tyr
2042–2046	**GAAAA**	GAAAA	GAAAA	TTTTC	GAAAA
2050	**A**	A	A	C	A

[a] In *Y. enterocolitica* serotype O13 and O21 strains and 8081 (serotype O8), *fyuA* nucleotide sequences have acquired an A-to-G silent transition at bp 1084; in *Y. enterocolitica* serotype O20 strains, *fyuA* has acquired a G-to-A silent transition at bp 267; in *Y. enterocolitica* serotype O13, O20, and O21 strains, *fyuA* nucleotide sequences have also acquired a T-to-C silent transition at bp 1770. Mutated nucleotides are shown in boldfaced letters.

[b] *fyuA* nucleotide sequence of *E. coli* CA42 has an inserted thymine nucleotide at bp −33, but it is numbered the same as all of the other *fyuA* sequences.

DISCUSSION

Human pathogenic yersiniae can be divided into pesticin-sensitive and pesticin-resistant groups on the basis of sensitivity to the *Y. pestis* bacteriocin pesticin. In principle, this correlates with the existence of two groups of yersiniae, mouse lethal (or highly pathogenic) and mouse nonlethal (8, 11, 28). Strains from the mouse-lethal group are pesticin sensitive, with the exception of non-serotype O1 *Y. pseudotuberculosis* isolates (6, 7, 29, 45). On the other hand, two rare *E. coli* clinical isolates, *E. coli* Phi and *E. coli* CA42, are known to be pesticin sensitive (6, 18, 45). Nevertheless, pesticin sensitivity has been proved to correlate with the virulence of yersiniae (7, 26, 29).

In this study, we have shown that pesticin sensitivity in *Yersinia* and *E. coli* strains is determined by the presence of the FyuA yersiniabactin or pesticin receptor that is encoded by the *fyuA* gene, previously characterized in a *Y. enterocolitica* serotype O8 strain (40). The *fyuA* sequence was not detected in pesticin-resistant, mouse-virulent *Y. pseudotuberculosis* serotype O2, O3, O4A, O4B, and O5A strains (Table 1).

For *Y. enterocolitica* serotype O8 strains, it has been demonstrated that the pesticin receptor also functions as a receptor for the yersinia siderophore yersiniabactin that is part of an iron uptake system which is present only in mouse-lethal yersiniae (27, 29, 39). The ability of FyuA to take up the siderophore-iron complex in iron-deprived tissues of the host may explain its possible impact on the virulence of yersiniae. Pesticin itself, an antimicrobial substance, can hardly be assumed to be a virulence factor (5, 47); more likely, it is a factor which allows *Y. pestis* to compete against other highly pathogenic *Yersinia* spp. or to eliminate immunogenic nonpesticinogenic avirulent mutants of *Y. pestis.*

From the recent data of Fetherston et al. (19, 20) and Carniel et al. (9, 10), it can be concluded that the genes that

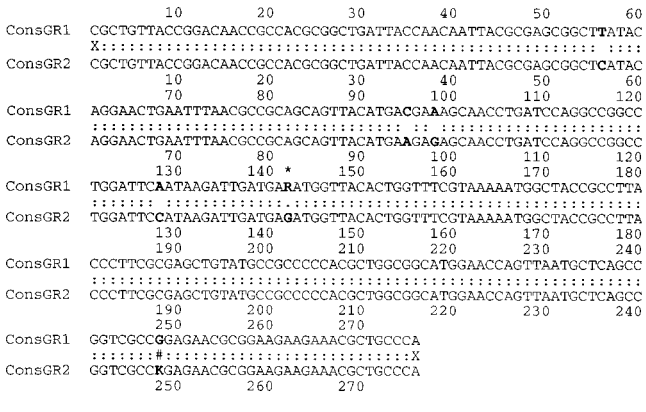

FIG. 4. Alignment of 276-bp *irp2* DNA sequence fragment of *irp2*-positive microorganisms. ConsGR1, *irp2* consensus sequence of *Y. enterocolitica* group strains, including *Y. enterocolitica* serotype O8 (WA-314 and 8081), O13, O20, and O21 strains. ConsGR2, *irp2* consensus sequence of *Y. pseudotuberculosis* group strains, including *Y. pseudotuberculosis* serotype O1 and O3, *Y. pestis* 6/69, and *E. coli* Phi, CA42, K49, and K235 strains. *, R = A in all *Y. enterocolitica* biotype 1B strains, except *Y. enterocolitica* 8081 (serotype O8), in which R = G. #, K = T in *Y. pestis* and *Y. pseudotuberculosis* serotype O1 and O3 strains and K = G in *E. coli* Phi, CA42, K49, and K235.

encode the pesticin receptor and the 190-kDa, iron-regulated HMWP2 (8) should be located within a 33- to 41-kb region of the pigmentation (*pgm*) locus of *Y. pestis*. The *pgm* locus consists of a large (102-kb) unstable chromosomal fragment that is flanked by two copies of the IS*100* insertion sequence in *Y. pestis* and may be responsible for the high spontaneous deletion frequency observed in *Y. pestis* (19, 20). Spontaneous deletions of a 35-kb chromosomal fragment that carries the *irp2* gene (encoding HMWP2) were also observed in nonpigment-sorbing *Y. pseudotuberculosis* strains (9, 10), in which this region is flanked by only one copy of IS*100* (20). IS*100* is absent from *Y. enterocolitica* serotype O8 strains (20), but we have identified a new 1,353-bp insertion sequence, designated IS*1328*, one copy of which is located 493 bp downstream of the *fyuA* gene in an AT-rich region (data not shown). IS*1328* might endow the *pgm* locus or part of it with features of a mobile DNA element, thus supporting genetic exchange of the *fyuA-irp2* gene cluster. Indeed, we found *irp2* sequences in all pesticin-sensitive yersiniae and also, to our surprise, in *fyuA*-positive *E. coli* strains. Thus, these results are consistent with genetic linkage of the *fyuA-irp2* gene cluster.

On the other hand, we were also able to detect *irp2* sequences in some pesticin-resistant, FyuA-negative, serotype O3 *Y. pseudotuberculosis* strains, which is in agreement with a recent report (14). *Y. enterocolitica* WA-3 (*irp2*$^+$) is a spontaneous pesticin-resistant mutant which has acquired a deletion of the *fyuA* gene, thus possibly explaining the appearance of *irp2*-positive, *fyuA*-negative *Y. pseudotuberculosis* serotype O3 strains.

Considering that different phenotypic patterns have been observed for *Y. pestis pgm* deletion mutants, it is conceivable that the genes of the *pgm* locus can be deleted separately or sequentially (33), resulting in a Pgm$^-$ Pst^r HMWP2$^+$ phenotype (typical for *Y. pseudotuberculosis* serotype O3 strains) or a Pgm$^-$ Pst^s HMWP2$^+$ phenotype (typical for *Y. pseudotuberculosis* serotype O1 and *Y. enterocolitica* biotype 1B strains).

The *pgm* locus of *Y. pestis* consists of several iron-repressible genes (e.g., *irpB*, *-C*, and *-D*) and includes *fyuA* and *irp2* (19, 20). As has been shown, *fyuA* is implicated in the expression of virulence because it encodes the yersiniabactin receptor and thus supports iron acquisition by the pathogen within the host (29, 39). Moreover, there is some evidence that *irp2* encodes enterobactin synthetase component F (homologous to EntF of *E. coli*), which might be also involved in yersiniabactin synthesis (23). Therefore, it is reasonable to assume that the *pgm* locus or the *fyuA-irp2* gene cluster has spread horizontally in some *Enterobacteriaceae* species by adaptive gene transfer from a common ancestor to improve the fitness of the recipient to its specific ecological niche. However, we cannot exclude the presence of another (or even several) siderophore uptake system in pathogenic *Yersinia* spp. (27, 37), at least not in nonserotype O1 strains of *Y. pseudotuberculosis.*

The possibility of lateral *fyuA-irp2* gene transfer is supported by the extremely high homologies of the *fyuA* (98.5 to 99.9%) and *irp2* sequences (97.8 to 100%). It turns out that the homology between *fyuA* and *irp2* genes from rather distinct *Enterobacteriaceae* genera (*Yersinia* and *Escherichia*) exceeds 98%, in contrast to 63% homology for their tRNAs (4) or 40% relatedness for total DNA among *Yersinia* species (only *Y. pestis* and *Y. pseudotuberculosis* DNAs are 90% or more interrelated) (2). The G+C content was found to be 56.2 mol% for *fyuA* and 59.8 mol% for *irp2* (23), thus exceeding the normal overall G+C content of 46 to 50 mol% for the *Yersinia* chromosome and 48 to 52 mol% for the *E. coli* chromosome (2).

On the basis of observed local differences in highly conserved *fyuA* and *irp2* sequences, two evolutionary lineages can be established, the *Y. enterocolitica* type (includes all biogroup 1B strains [serotypes O8, O13, O20, and O21]) and the *Y. pseudotuberculosis* type (*Y. pseudotuberculosis* serotype O1 strains, *Y. pestis*, *E. coli* Phi, CA42, K49, and K235, and *irp2*-positive *Y. pseudotuberculosis* serotype O3 strains).

Y. enterocolitica-type *fyuA* genes have the largest number of nucleotide substitutions. Even though most of these nucleotide substitutions are silent, some of them can lead to conformational changes in the FyuA protein structure in domains that interact with TonB protein (TonB box and the conserved carboxyl end) (30, 42, 48). These substitutions in TonB-interacting regions (34, 36) in *Y. enterocolitica* can influence the structure of the pesticin receptor, thus leading to differences in sensitivity to pesticin action (32). On the other hand, these substitutions may be required for efficient interaction with TonB, thus reflecting differences in TonB organization between *Y. enterocolitica* and *E. coli* (35).

Inactivation of the *fyuA* gene in *Y. enterocolitica* or the *irp2* gene in *Y. pseudotuberculosis* or deletion of the *pgm* locus in *Y. pestis* results in reduced virulence for mice (9, 14, 20, 29). These results strongly suggest that the *pgm* locus can be considered an unstable, perhaps mobile, genomic pathogenicity island of yersiniae, similar to what has recently been described for uropathogenic *E. coli* (3, 25). The extremely high homologies of the *fyuA* and *irp2* genes of pesticin-sensitive bacteria favor the idea of lateral transfer of the *fyuA-irp2* gene cluster from a common ancestor.

The *fyuA-irp2* gene cluster has been established in *Yersinia* spp., thus supplying them with an additional efficient iron uptake mechanism via siderophore, or has simply been maintained in *E. coli* isolates as a possibly neutral gene block. However, it looks as if the *fyuA* gene in *E. coli* has been exposed to the same degree of selection pressure as the *fyuA* genes in *Y. pestis* and *Y. pseudotuberculosis* have. At present, we cannot explain the high homologies of *fyuA* genes among *E. coli* and *Y. pestis* strains in terms of selection for virulence, but it is also possible that the *fyuA-irp2* gene cluster contains other genes by which these pesticin-sensitive *E. coli* strains gain greater fitness for their ecological niche.

Today most data favor the idea that pathogenicity characters are acquired by bacteria as a result of horizontal transfer of gene blocks, specifying different virulence determinants (16), not slow adaptation. This appears to be the case for the *fyuA-irp2* pathogenicity gene cluster.

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