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The Yersiniabactin Biosynthetic Gene Cluster of *Yersinia enterocolitica*: Organization and Siderophore-Dependent Regulation

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The ability to synthesize and uptake the *Yersinia* siderophore yersiniabactin is a hallmark of the highly pathogenic, mouse-lethal species *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* 1B. We have identified four genes, *irp1*, *irp3*, *irp4*, and *irp5*, on a 13-kb chromosomal DNA fragment of *Y. enterocolitica* O8, WA-314. These genes constitute the yersiniabactin biosynthetic gene cluster together with the previously defined *irp2*. The *irp1* gene consists of 9,486 bp capable of encoding a 3,161-amino-acid high-molecular-weight protein 1 (HMWP1) polypeptide with a predicted mass of 384.6 kDa. The first 3,000 bp of *irp1* show similarity to the corresponding regions of the polyketide synthase genes of *Bacillus subtilis* and *Streptomyces antibioticus*. The remaining part of *irp1* is most similar to *irp2*, encoding HMWP2, which might be the reason for immunological cross-reactivity of the two polypeptides. Irp4 was found to have 41.7% similarity to thioesterase-like protein of the anguibactin biosynthetic genes of *Vibrio anguillarum*. Irp5 shows 41% similarity to EntE, the 2,3-dihydroxybenzoic acid-activating enzyme utilized in enterobactin synthesis of *Escherichia coli*. Irp4 and Irp5 are nearly identical to YbtT and YbtE, recently identified in *Y. pestis*. *irp3* has no similarity to any known gene. Inactivation of either *irp1* or *irp2* abrogates yersiniabactin synthesis. Mutations in *irp1* or *fyuA* (encoding yersiniabactin/pesticin receptor) result in downregulation of *irp2* that can be upregulated by the addition of yersiniabactin. A FyuA-green fluorescent protein translational fusion was downregulated in an *irp1* mutant. Upregulation was achieved by addition of yersiniabactin but not desferal, pesticin, or pyochelin, which indicates high specificity of the FyuA receptor and autoregulation of genes involved in synthesis and uptake of yersiniabactin.

The genus *Yersinia* contains at least 11 species, 3 of which are enteropathogenic for humans. *Yersinia pestis* is the agent of bubonic plague, while *Y. pseudotuberculosis* and *Y. enterocolitica* are pathogenic for humans. *Y. enterocolitica* causes a broad range of diseases ranging from acute bowel disease to extraintestinal manifestations such as reactive arthritis and uveitis. Human-pathogenic *Yersinia* species can be divided into highly pathogenic *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* biotype 1B (so-called American serotypes), which are mouse lethal at low doses, and low-pathogenic *Y. enterocolitica* biotypes 2 to 5 (so-called European serotypes), which are not mouse lethal at low doses (10).

A prerequisite for any expression of pathogenicity by *Yersinia* is the presence of a 70-kb pYV virulence plasmid that is found in high- and low-pathogenic strains (4, 21, 27). Differences in mouse virulence seem to be chromosomally determined. Highly pathogenic strains possess a chromosomal cluster of iron-regulated genes designated the high-pathogenicity island (HPI). This island is absent in low-pathogenic or nonpathogenic strains and was found to be unstable in *Yersinia* strains. Its loss leads to a marked reduction in mouse virulence (36).

Two proteins encoded by iron-repressible genes have been detected only in highly pathogenic *Yersinia* strains, being putatively located on the HPI: HMWP1 (high-molecular-weight protein 1; 260 kDa, encoded by *irp1*) and HMWP2 (190 kDa, encoded by *irp2*) (9, 15). Inactivation of *irp2* in *Y. pseudotuberculosis* results in a considerable reduction of mouse virulence (8). HMWPs are suspected to be important for siderophore yersiniabactin production and therefore involved in the expression of a CAS (chrom azurol S ferric ion indicator dye)-positive phenotype in highly pathogenic *Yersinia* strains (31). The receptor of yersiniabactin, FyuA (ferric yersiniabactin uptake), is a receptor with dual function: it is a receptor of the siderophore and a receptor of *Y. pestis* bacteriocin pesticin. Thus, highly pathogenic strains are pesticin sensitive (Pst^s) because of such a dual nature of FyuA (44). Yersiniabactin and FyuA were shown to be produced only by mouse-lethal strains (32).

In *Y. pestis*, the *fyuA* gene, the *irp2* gene, and the *hms* locus (encoding hemin storage) are located on a 102-kb fragment designated the *pgm* (pigmentation) locus. This fragment is flanked by two copies of the insertion sequence (IS) element IS*100* (23, 41), which might be the reason for frequent deletions of the *pgm* locus. *ybtA*, a gene encoding a protein belonging to the AraC family of transcriptional regulators, was recently detected upstream the *irp2* gene in *Y. pestis*. YbtA is believed to be a transcriptional activator of the yersiniabactin receptor and of the siderophore biosynthetic genes (22). Bearden et al. (3) have identified an approximately 22-kb region of the *pgm* locus of *Y. pestis* which encodes several iron-regulated proteins. Some of them (YbtT and YbtE) were shown to be involved in the biosynthesis of a putative siderophore of *Y. pestis*.

The HPI of *Y. enterocolitica* contains the *fyuA* and *irp2* genes but does not harbor genes for the hemin storage (24). This locus is much more stable than the *pgm* locus of *Y. pestis*. No flanking IS*100* elements, but at least two IS elements, IS*1328* and IS*1400*, were identified downstream *fyuA* in *Y. enteroco-*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or phenotype	Reference(s) or source
Strains		
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrArelA1* Δ(*lacZYA-argF*)*U169* (ϕ80*lacZ*Δ*M15*)	30
Phi	Pst^s	19
S17-1 λ	*pir*$^+$ *tra*$^+$	54, 39
Y. enterocolitica		
WA-C	Plasmidless derivative of strain WA-134, serotype O8. Spontaneous Nal^r mutant	31
WA-C *irp2*	WA-C with pGPIRP2 inserted into *irp2* gene	This study
WA-CS	Spontaneous Sm^r mutant derived from WA-C	This study
WA *fyuA*	*fyuA* mutant of WA-C, Pst^r	44
WA-CS *irp1*::Kan^r	Derivative of WA-CS, Nal^r Sm^r Km^r	This study
8081	Serotype O8	42
H1852 *fyuA*	Derivative of WA-C, *fur fyuA* mutant of WA-C	This study
Y5.27	Clinical isolate, serotype O5.27	31
Y-96-C	Clinical isolate, serotype O9	31
Y-108-C	Clinical isolate, serotype O3	31
Y. pseudotuberculosis		
346	*irp2*$^+$ serotype O3	S. Aleksic
201	*irp2* serotype O3	S. Aleksic
PB1	Serotype O1	R. R. Brubaker
Y. pestis		
KIM	Lcr$^-$ Pgm$^+$	R. R. Brubaker
KIM Δ*pgm*	Lcr$^-$ ΔPgm	This study
KUMA	Lcr$^-$ Pgm$^+$	R. R. Brubaker
EV76	Pesticin-producing strain	R. R. Brubaker
Plasmids		
pLAFR2	Tc^r Mob$^+$	25
17A11	pLAFR2 carrying a 23-kb insert of WA-C chromosomal DNA, Tc^r	44
pBluescript KS II	Cloning vector, Ap^r	Stratagene
pKAS 32	Cloning vector with *rpsL* gene	55
pKAS-1SKan	pKAS 32 containing *irp1* *Eco*RI-2 fragment with a kanamycin cassette introduced into *Sal*I site	This study
pKAS-E1Kan	pKAS 32 containing *Eco*RI/*Sal*I fragment of *irp1* with a kanamycin cassette (without transcriptional terminator) inserted into the *Eco*RV site	This study
pGP704	Cloning vector, Ap^r	39
pGP-CAT	Chloramphenicol cassette inserted into *Pst*I site of pGP704, Cm^r	46
pGPIRP2	600-bp PCR fragment of *irp2* inserted into pGP-CAT vector	This study
pUC-4K	pUC vector containing kanamycin cassette from Tn*903*, Ap^r Kan^r	60
pSB 315	Containing kanamycin cassette without transcriptional terminator, Ap^r Kan^r	27
pACYC 184	Cloning vector, Cm^r	11
pCJG3.3N	pACYC 184 with *fyuA-gfp* mut3	This study
pGFP mut3	pKS with cDNA of the mutated GFP under the *lac* promoter	12
SuperCos1	Cloning vector lacking *Sal*I restriction site, Ap^r Neo^r	Stratagene

litica O8 (7, 43). *Irp2* and *fyuA* are separated by approximately 12 kb. This fragment may contain additional *irp* genes involved in siderophore synthesis, including *irp1* (encoding HMWP1). In this study, we have determined the nucleotide sequence of the *irp1* to *irp5* genes of *Y. enterocolitica* O8, shown their involvement in yersiniabactin biosynthesis, and demonstrated the siderophore-directed regulation of yersiniabactin synthesis and receptor genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the study are listed in Table 1. Strains were grown in Luria-Bertani (LB) broth or on LB agar plates (Difco Laboratories, Detroit, Mich.) at 28°C (*Yersinia*) or 37°C (*Escherichia coli*). Iron-deficient medium (NBD) was made by adding 200 μM α-α-dipyridyl (Sigma, St. Louis, Mo.) to NB medium (nutrient broth [Difco] with 5 g of NaCl per liter as described previously [44]).

WA-CS is a derivative of WA-C (*Y. enterocolitica* serotype O8 WA-314, plasmid cured). Spontaneous streptomycin-resistant (Sm^r) colonies of nalidix acid-resistant (Nal^r) WA-C were isolated by increasing streptomycin concentrations in LB medium (with 10, 30, 50, 70, and 100 μg/ml). The resulting strain was designated WA-CS. Strain KIM Δ*pgm* was isolated as a spontaneous mutant unable to accumulate Congo red dye on LB medium containing 50 μg of Congo red per ml.

DNA manipulation. Bacterial chromosomal DNA was isolated by the method of Davis et al. (14). A gene bank was prepared from *Y. enterocolitica* WA-314 serotype O8. The chromosomal DNA was partially digested with *Sau*3A and ligated into the *Bam*HI site of vector pLAFR2 (25).

Southern blot hybridizations (56) were performed with digoxigenin (DIG)-labeled PCR probes, using the following primers: P242 (′5-AAGGATTCGCTG TTACCGGAC-3′) and P505 (′5-ATTCGTCGGGCAGCGTTTCTTCT-3′) for the start of *irp2*, P4801 (′5-ATTGCCGATCTGGACCTC-3′) and P5206 (′5-A TCTGGATTGGCGACTGTAG-3′) for the end of *irp2*, i8513 (′5-TGAATCGC GGGTGTCTTATGC-3′) and i8730 (′5-TCCCTCAATAAAGCCCACGCT-3′) for *irp1* P161 (′5-CAACATCGTCACCCAGCAG-3′) and P191 (′5-CGCAGTA GGCACGATGTTGTA-3′) for *fyuA*, and R299 (′5-TTTACAATTACACACCC TCAA-3′) and P732 (′5-CTGGGAGATGGGAAAAACTAC-3′) for IS*1328*, plus DIG-11-dUTP according to the Boehringer Mannheim Biochemica protocol.

DNA sequencing and sequence comparison. The subcloned fragments *Eco*RI-2 and -3 from cosmid 17A11 (Fig. 1) were treated with exonuclease III (Nested Deletion kit; Pharmacia Biotech). Vector primers for templates generated by exonuclease digestion were used.

Sequence-specific oligonucleotides were synthesized for nonoverlapping re-

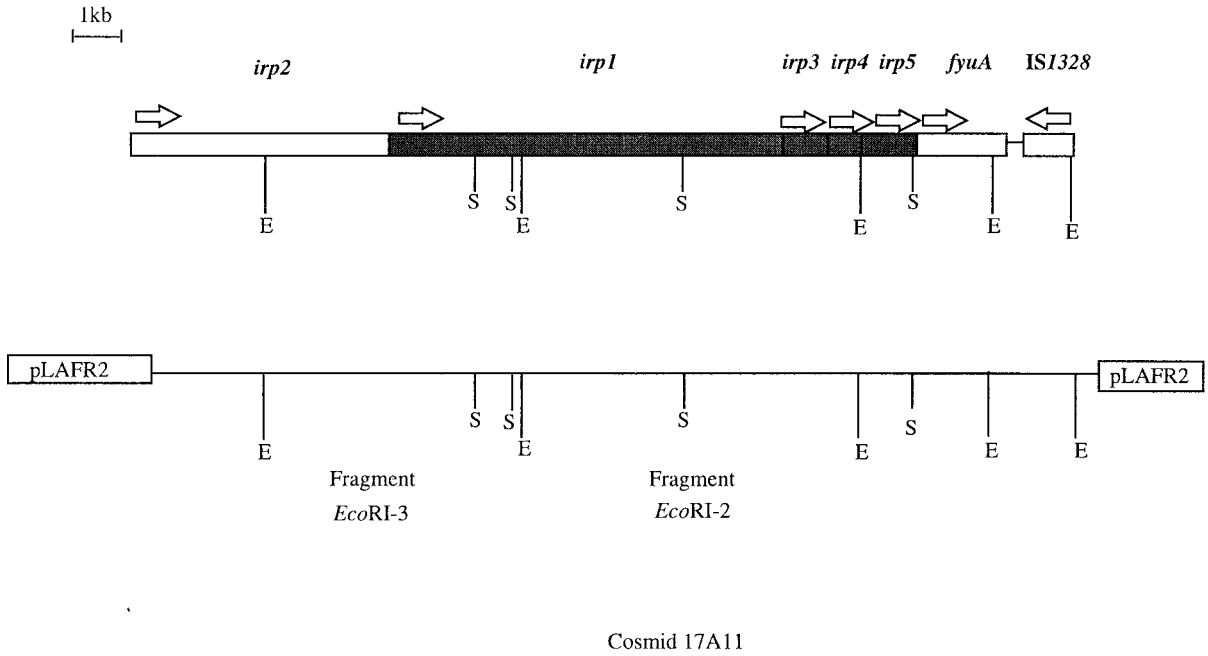

FIG. 1. Genetic organization of the *Y. enterocolitica* O8 WA-314 *irp2-fyuA* gene cluster. The genes are depicted as boxes. Arrows above indicate the direction of transcription. E, *Eco*RI; S, *Sal*I.

gions and for the region downstream fragment *Eco*RI-2 and upstream *fyuA* (primer walking). DNA sequencing was performed by the chain-terminating method with model 373A and 377 DNA sequencers (ABI Prism; Perkin-Elmer). The sequences were analyzed and aligned with the HIBIO Mac DNASIS program (Hitachi Software Engineering Co.) and with the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison).

PCR conditions. PCR amplifications were performed in an automated thermal cycler (TRIO Thermoblock; Biometra or GeneAmp PCR System 2400; Perkin-Elmer) as described by Saiki et al. (48) with *Taq*I polymerase and different pairs of oligonucleotides (Roth; Karlsruhe, Mannheim, Germany). The initial denaturation step (94°C, 7 min) was followed by 35 cycles of denaturing, annealing, and extension with one final extension step. Annealing and extension temperatures were set according to the primers used. PCR amplification products were separated in 1.6% agarose gels followed by purification with a QIAquick PCR purification kit or gel purified by using QIAquick gel extraction kit 250 (Qiagen GmbH, Hilden, Germany).

Comparison of the *irp1* sequences of different strains was performed by using primers i965 (5′-CATCGACGACAGGCAGGTAGG-3′, bp 965 to 986) and i1233 (5′-CGGTATGGTAAAGGACTCTC-3′, bp 1233 to 1253) for the beginning and primers i8513 (5′-TGAATCGCGGGTGTCTTATGC-3′, bp 8513 to 8534) and i8730 (5′-TCCCTCAATAAAGCCCACGCT-3′, bp 8730 to 8751) for the end of *irp1*.

Construction of *irp1* and *ipr2* mutants. The *Eco*RI-2 fragment from cosmid 17A11 (Fig. 1) was ligated into the *Eco*RI site of vector SuperCos1. Fragment *Eco*RI-2 harbors a *Sal*I site in the open reading frame (ORF) of *irp1*. A kanamycin cassette containing a *Sal*I fragment from plasmid pUC-4K was inserted into it. Fragment *Eco*RI-2 of *irp1* with the kanamycin cassette was ligated into the pKAS 32 suicide vector (designated pKAS-1SKan). pKAS 32 contains the *rpsL* gene, which encodes the S12 protein of the ribosomes (55). Insertion of the suicide vector into the chromosome results in a Sm^s phenotype of a formerly Sm^r strain. Kan^r (kanamycin resistance) Sm^r arose after an allelic exchange (double crossover) had taken place and the vector was lost. The construct was transformed into *E. coli* S17-1 λ pir^+ tra^+ (39, 54) followed by mobilization into WA-CS. Mutants were selected on agar plates containing kanamycin (40 μg/ml), streptomycin (100 μg/ml), and nalidixic acid (100 μg/ml), and the presence of the kanamycin cassette in *irp1* was confirmed by Southern hybridization. To exclude a polar effect on the CAS phenotype, we created a second *irp1* mutant by using a kanamycin cassette without transcriptional terminator. The *Eco*RI/*Sal*I fragment of *irp1* harboring an *Eco*RV cutting site (182 bases downstream of the *Eco*RI site) was inserted into the pKS vector, and the kanamycin cassette from pSB 315 cut by *Hin*dII was ligated into the *Eco*RV restriction site. The construct was excised with *Kpn*I/*Sac*I and inserted into the pKAS 32 suicide vector (resulting in pKAS-E1Kan) followed by mobilization and selection as described above.

Mutagenesis of *irp2* was performed as described previously (46). Briefly, an internal PCR product of the *irp2* gene from *Y. enterocolitica* O8 strain WA-C (primers UP *irp2-sac1* [5′-CTCGAGCTCAAGGATTCGCTGTTACCGGAC-3′] and LP *irp2-sac1* [5′-CTCGAGCTCTCGTCGGGCAGCGTTTCTTCT-3′]) was ligated into the *Sac*I site of the suicide vector pGP-CAT and transformed into *E. coli* S17-1 λ pir^+ tra^+, generating pGPIRP2. The suicide hybrid plasmid pGPIRP2 was integrated into the *irp2* gene of WA-C following conjugation and homologous recombination, giving rise to the *Y. enterocolitica* mutant strain WA-C *irp2*. The correct insertion of pGPIRP2 into the chromosomal DNA was confirmed by Southern hybridization.

FyuA-GFP reporter gene studies. We translationally fused 267 amino acids (aa) of FyuA (including the upstream regulatory sequences and the putative YbtA binding site) and the product of the reporter gene *gfp* (encoding green fluorescent protein [GFP]) mut3 by using standard PCR cloning procedures and primers with designed restriction sites (*Hin*dIII-*Bam*HI [FyuA] and *Bam*HI-*Sal*I [GFP mut3]). The resultant plasmid, pCJG3.3N, was transferred into WA-C and WA-CS *irp1*::Kan^r by electroporation.

Flow cytometric measurements were performed with a Coulter Epics Flow cytometer. An argon 488-nm laser was used. Bacteria were detected by side scatter as described by Russo-Marie et al. (47). The scale was logarithmic, and fluorescence data and scatter data were collected for 50,000 bacteria.

Growth experiments: feeding assay with yersiniabactin containing culture supernatant, desferrioxamine, purified yersiniabactin, and pyochelin. *Y. enterocolitica* mutant H1852 *fur fyuA* (siderophore hyperproducer) was cultivated aerobically in iron-deficient NBD medium for 12 h at 28°C (29). After centrifugation, the supernatant containing siderophore was sterilized by filtration and used for feeding experiments. Desferrioxamine (Desferal) was obtained from Ciby Geigy. Purified yersiniabactin and pyochelin preparations used for final confirming experiments were kindly provided by R. Reissbrodt (Wernigerode, Germany) and H. Budzikiewicz (Cologne, Germany).

Pesticin assay. Pesticin-producing strain *Y. pestis* EV76 was grown overnight at 26°C, and pesticin production was induced by mitomycin C (0.3 μg ml^{-1}) for an additional 16 h. Cells were collected by centrifugation, and the supernatant was used as a crude pesticin preparation after sterilization with 0.1% chloroform (35). Sensitivity to pesticin was monitored by serial dilution of the supernatant (1:2) on mid-log-phase bacterial cultures (10^6 microorganisms) in 0.6% LB agar used as an overlay (double-layer technique) with 1.2% LB agar as a support. Plates were incubated at 37°C for 18 h.

Screening for iron-chelating compounds. Strains to be tested were plated on CAS agar (52) and incubated for 2 days at 26°C. A clearly visible red-orange halo around bacterial colonies was indicative of siderophore production (i.e., colonies were CAS positive).

SDS-PAGE and Western blotting. The bacteria were cultured under iron-limiting conditions in NBD broth, centrifuged, washed, and solubilized by boiling in Laemmli buffer (total cell lysate) (38). Equal amounts of all strains (50 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel at a constant current of 40 mA. The gel was stained with Roti-Blue (Roth) or electroblotted to nitrocellu-

lose membranes (BA85; Schleicher & Schüll, Inc., Dasserl, Germany) as described previously (33). HMWP-specific antibodies were kindly provided by E. Carniel (Institut Pasteur, Paris, France). These antibodies had been obtained by using a purified HMWP fraction to immunize BALB/c mice. They specifically recognized the two HMWPs. Antibodies directed against one HMWP also recognize the other one (6).

Nucleotide sequence accession number. The sequence determined was deposited at the EMBL/GenBank database under accession no. Y12527.

RESULTS

Cloning of *irp1* and nucleotide sequence determination. A *Y. enterocolitica* WA-C gene bank was screened for the presence of cosmids harboring the *irp2-fyuA* fragment by Southern hybridization. One of the cosmids, 17A11 with an insert of approximately 23 kb, hybridized with the *irp2* terminal probe, *fyuA*, and the IS*1328* element, indicating that this cosmid carries the DNA fragment covering the region between *irp2* and *fyuA*. 17A11 was digested with *Eco*RI, resulting in six fragments of 23 (pLAFR2 vector portion), 8.2, 6.2, 3.3, 2.6, and 2.1 kb. The 3.3-kb *Eco*RI fragment hybridized with the *fyuA* probe, the 2.6-kb *Eco*RI fragment hybridized with the *irp2* probe, and the 2.1-kb *Eco*RI fragment hybridized with the IS*1328* probe. Double digestion with *Eco*RI and *Sal*I revealed a physical restriction map of the 23-kb insert shown in Fig. 1. The 6.2-kb *Eco*RI fragment (designated fragment *Eco*RI-3) hybridized with the *irp2* probe (bp 4581 to 5007 of *irp2*) downstream the *Eco*RI site. The 8.2-kb *Eco*RI fragment (designated fragment *Eco*RI-2) did not hybridize with any of the probes. It has been postulated that *irp1* constitutes one operon with *irp2* (8). We assumed that fragments *Eco*RI-2 and -3 comprise most likely the *irp1* gene encoding HMWP1 with the size of 240 kDa. Therefore, both fragments were subcloned into the pKS vector and sequenced.

Fragment *Eco*RI-3 contains the terminal portion of *irp2* downstream of the *Eco*RI site and the start of a new ORF 87 bp downstream the TAG stop codon of *irp2* (Fig. 1). The residual portion of the new defined ORF is located on fragment *Eco*RI-2. Taken together, the data suggest that this new ORF, which is likely to be *irp1*, consists of 9,486 nucleotides, encoding a 3,161-aa polypeptide of 384.6 kDa. The ORF has the same transcriptional direction as *irp2*. No ORF of significant length was found on the complementary strand. The G+C content of the sequence is approximately 60 mol%, higher than the value of 47 to 50 for *Yersinia* (5) but similar to that for *irp2*.

A palindrome of 29 bases (boldface) capable of forming a secondary stem-loop structure is located between *irp2* and *irp1* (**GGAACGCCATCGCGAACGCATGGCGTTCC**). The palindrome starts 23 nucleotides after the stop codon of *irp2* and lacks a poly(T) tail. Thus, it is unlikely that it is a transcriptional terminator. The GGA sequence located 6 bp upstream the first ATG codon of *irp1* may serve as a weak ribosome-binding site. No promoter-operator structure could be identified upstream of *irp1*.

Another ORF, designated *irp3*, starts directly downstream *irp1*. The GGAG sequence 10 bp upstream the start codon can be considered a ribosome-binding site. *irp3* consists of 1,098 nucleotides, encoding a 365-aa polypeptide of 40.7 kDa. *irp3* has the same orientation as *irp1* and *irp2*. The G+C content is around 60 mol%, which is in the same range as values for *irp1* and *irp2*.

Two more ORFs, of 804 bp (designated *irp4*) and 1,578 bp (designated *irp5*), could be identified between *irp3* and *fyuA*. These genes have the same transcriptional direction as *irp2* and similar values for G+C content.

Taken together, data show that the *irp* gene cluster of *Y. enterocolitica* comprises five genes located upstream *fyuA* in the following order: *irp2-irp1-irp3-irp4-irp5* (Fig. 1). *irp1* to *irp3*, and *irp3* and *irp4*, are contiguous; *irp4* and *irp5* are divided by three bases; *irp2* and *irp1* are separated by 87 bases. The gene order was confirmed by comparative Southern hybridization of cosmid 17A11 and WA-C chromosomal DNA digested with *Eco*RI and using PCR products corresponding to *irp1* (bases 8513 to 8730) and *irp2* (bases 4581 to 5007) as probes. Either probe hybridized to bands of the same size in digests of cosmid 17A11 and WA-C DNA (data not shown).

Motif1

```
aa 1960   WNQLIARHDMLRMVVDADGQQRVLGTTPE 1988  HMWP1
          ||.|||||||| ||  ||||.||: ||.
aa 1540   WNRLIARHDMLRAVV.LDGQQQVLEQTPP 1567  HMWP2
```

Motif2

```
aa 2174  FTLNLTFFNRQPIHPQINQLIGDFTSVTLVD 2204  HMWP1
         ||||||:|:|.|:||||||::|||||: |:.
aa 1747  FTLNLTLFDRRPLHPQINQILGDFTSLMLLS 1778  HMWP2
```

Motif3

```
aa 2285  VFTQTPQVWLDHQVMESDGELMFSWY 2310  HMWP1
          . ||||||||||| ||:|||.|.|
aa 1852  GISQTPQVWLDHQVYESEGELRFNWD 1877  HMWP2
```

FIG. 2. Common amino acids motifs found in HMWP1 and HMWP2.

DNA and protein sequence homology. To identify similarities of *irp1* to *irp5* and their deduced polypeptides to known sequences, a search in the EMBL gene data library was performed with the FastA program. The *irp1* DNA sequence has highest identity to a cosmid from *Mycobacterium tuberculosis* (57.5% identity in a 1,049-bp overlap [bases 490 to 1530]; unpublished, accession no. Z83857), 53.1% identity over 971 bp (bp 187 to 1131) to the polyketide synthase gene of *Bacillus subtilis* W168 (53), 52.9% identity in a 2,763-bp overlap (bp 1 to 2678) to the *eryA* gene of *Saccharopolyspora erythraea* (17), 51.4% identity over 1,713 bp (bp 195 to 1866) to the polyketide synthase gene of *Streptomyces antibioticus* (58), 64.2% identity in 162 bp (bp 175 to 335) to the polyketide immunosuppressant gene of *Streptomyces hygroscopicus* (1, 40, 51), and 52.9% identity in a 1,481-bp overlap (bp 5716 to 7132) to *irp2* of *Y. enterocolitica* (28). Interestingly, similarities to all of these related sequences are located within the first 3,000 nucleotides, whereas the following 6.3 kb show similarity only to *irp2*. A potential β-ketoacyl synthase active site could be identified in HMWP1 between aa 184 and 210. β-Ketoacyl-ACP (acyl carrier protein) synthase is the enzyme that catalyzes the condensation of malonyl-ACP with the growing fatty acid chain and is also found as a component in polyketide antibiotic synthases.

irp4 and *irp5* were found to be 97.3 and 98.3% identical to *ybt*T (0.8 kb) and *ybt*E (1.5 kb), recently described for *Y. pestis* (3). Deduced proteins have 41.7% identity (Irp4) with a thioesterase-like protein located in the anguibactin biosynthetic gene cluster of *Vibrio anguillarum* (20) and 41% amino acid sequence identity (Irp5) with EntE, the 2,3-dihydroxybenzoic acid-activating enzyme utilized in the enterobactin biosynthetic pathway of *E. coli* (57). *irp3* has no significant similarity to any known gene.

Amino acid sequence comparison of the polypeptides encoded by *irp1* and *irp2* showed three highly conserved motifs (Fig. 2). The presence of such motifs might be the reason for the cross-reactivity of HMWP1 and HMWP2 which was found even with monoclonal antibodies raised against the HMW proteins (37).

Presence of *irp1* in various *Yersinia* species and *E. coli*. The presence of *irp1* in various *Yersinia* and *E. coli* isolates was

TABLE 2. Results of Southern hybridization with *irp1*, *irp2*, and *fyuA* probes

Strain	*irp1*	*irp2*	*fyuA*
Y. enterocolitica			
WA-C	+	+	+
8081	+	+	+
Y5.27	−	−	−
Y-96-C	−	−	−
Y-108-C	−	−	−
Y. pseudotuberculosis			
346	+	+	−
201	−	−	−
PB1	+	+	+
Y. pestis			
KIM	+	+	+
KUMA	+	+	+
KIM Δ*pgm*	−	−	−
E. coli			
DH5α	−	−	−
Phi	+	+	+

tested by Southern hybridization. The chromosomal DNAs of various strains (Table 2) were digested with *Eco*RI, and Southern hybridization was performed with an *irp1* probe (corresponding to bp 7901 to 8139). As expected, *irp2*/*fyuA*-negative strains were also devoid of *irp1*. In *irp2*/*fyuA*-positive strains (*Y. enterocolitica* WA-CS and 8081, *Y. pestis* KIM and KUMA, *Y. pseudotuberculosis* PB1 and 346, and *E. coli* Phi), a band that hybridized with the *irp1* probe was detected (Fig. 3).

It was shown that *irp2* is highly conserved between different *Yersinia* species (45). We analyzed the degree of variability of *irp1* in the *irp1*-positive strains *Y. enterocolitica* WA-CS and 8081, *Y. pestis* KIM and KUMA, *Y. pseudotuberculosis* PB1 and 346, and *E. coli* Phi. PCR was performed for the start (bp 965 to 1254) and end (bp 7901 to 8139) portions of *irp1*; 150 bases of these amplicons were sequenced in both directions (bases 1041 to 1191 and 7948 to 8098). Comparison between these sequences and the *irp1* sequence obtained for WA-C revealed 100% identity between all *irp1*-positive strains over bases 7948 to 8098. Four base substitutions in the amplicon (bp 1041 to 1191) were found between *irp1* sequences of *Y. enterocolitica* (WA-C and 8081) and *Y. pseudotuberculosis*, *Y. pestis* and *E. coli* Phi (Fig. 4).

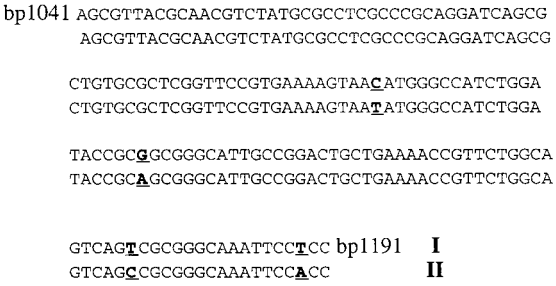

FIG. 4. Comparison of the region from bp 1041 to 1191 of *irp1* in *Y. enterocolitica* 8081, *Y. enterocolitica* WA-CS (I), *Y. pestis* KUMA, *Y. pestis* KIM, *Y. pseudotuberculosis* 346, *Y. pseudotuberculosis* PB1, and *E. coli* Phi (II). Nonmatching bases are boldfaced and underlined.

***irp1*- and *irp2*-encoded proteins are involved in yersiniabactin synthesis.** The possible relationship between the CAS phenotype and HMWP production was analyzed by mutagenesis of gene *irp1*. A kanamycin cassette was inserted into *irp1* gene of WA-CS, and allelic exchange was performed. The resulting mutant, WA-CS *irp1*::Kan^r, was tested on CAS agar and found to be CAS negative, indicating loss of yersiniabactin production (Fig. 5). The polar effect of the kanamycin cassette on the *irp* operon was ruled out by interruption of *irp1* with a kanamycin cassette lacking a transcriptional terminator. This mutant was also unable to form a halo on CAS agar. The same result was achieved by inserting the total suicide vector pGP CAT carrying a fragment of *irp2* (designated pGPIRP2) into the *irp2* gene by homologous recombination (data not shown). These results demonstrate that mutants disrupted in either *irp1* or *irp2* lost the ability to synthesize the siderophore yersiniabactin.

Yersiniabactin-dependent expression of the *irp* operon. To evaluate the effect of the *irp1* inactivation on the expression of HMWP1, a total-cell lysate of WA-CS and WA-CS *irp1*::Kan^r grown under iron-poor conditions (NBD medium) was subjected to SDS-PAGE. HMWP1 could not be detected in the mutant strain, while HMWP2 was still visible as a very faint

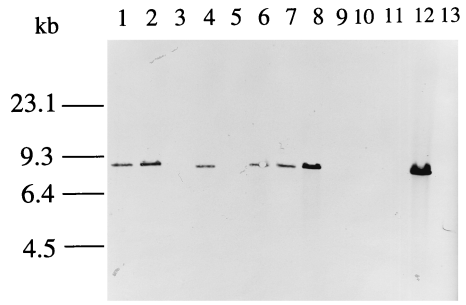

FIG. 3. Southern hybridization of chromosomal DNAs from *Yersinia* and *E. coli* strains with the *irp1* probe. The chromosomal DNA was digested with *Eco*RI, and the resulting fragments were separated on a 1% agarose gel prior to Southern blotting. Hybridization was performed with a DIG-labeled PCR probe generated with primers i8513 and i8730. Lane 1, *Y. pestis* KUMA; lane 2, *Y. pestis* KIM; lane 3, *Y. pestis* KIM Δ*pgm*; lane 4, *Y. pseudotuberculosis* 346; lane 5, *Y. pseudotuberculosis* 201; lane 6, *Y. pseudotuberculosis* PB1; lane 7, *Y. enterocolitica* 8081; lane 8, *Y. enterocolitica* WA-CS; lane 9, *Y. enterocolitica* Y5.27; lane 10, *Y. enterocolitica* Y-96-C; lane 11, *Y. enterocolitica* Y-108-C; lane 12, *E. coli* Phi; lane 13; *E. coli* DH5α.

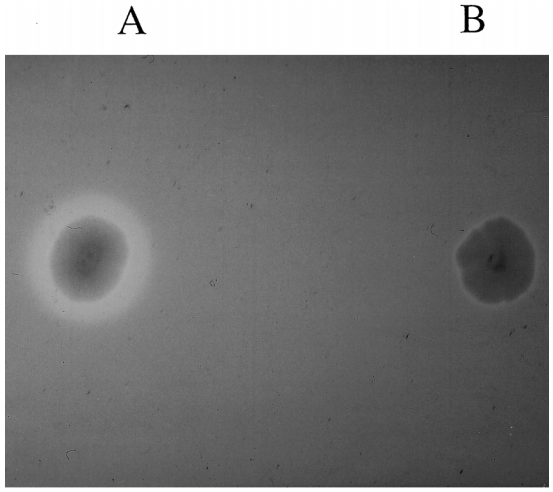

FIG. 5. CAS agar plate showing siderophore-producing *Y. enterocolitica* WA-CS (A; with halo) and mutant WA-CS *irp1*::Kan^r (B).

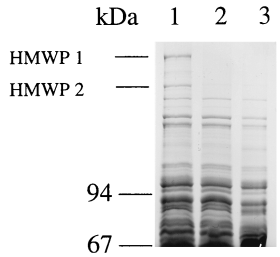

FIG. 6. Expression of HMWP1 and -2 in WA-CS *irp1*::Kan^r and WA *fyuA* mutants. SDS-PAGE (7.5% gel) of total-cell proteins from iron-starved strains WA-CS (lane 1), WA-CS *irp1*::Kan^r (lane 2), and WA *fyuA* (lane 3).

band after Roti-Blue staining (Fig. 6). The parent strain WA-CS expressed both HMWPs in comparable amounts. An additional weak protein band could be detected between HMWP1 and HMWP2. This band is thought to be a modified form of HMWP2 (5a) and reacts with the HMWP antibodies (Fig. 7). Production of HMWP1 and -2 was also decreased in an *fyuA* mutant impaired in its ability of ferric yersiniabactin uptake (Fig. 6). HMWP1 and HMWP2 were detected with anti-HMWP1 and anti-HMWP2 antisera in the wild-type strain but not in the mutant WA-CS *irp1*::Kan^r (Fig. 7).

The pesticin sensitivity mediated by the FyuA receptor was not impaired by *irp1* inactivation. The pesticin bactericidal titer was 1:512 for both strains, and no difference in the extent of sensitivity between the wild-type and mutant strains was detected.

To test the possibility that inactivation of *irp1* and subsequent failure in yersiniabactin production directly lead to downregulation of *irp2* expression, WA-CS and WA-CS *irp1*::Kan^r strains were grown in NBD iron-poor medium with and without addition of a culture supernatant containing yersiniabactin. A sublethal concentration of pesticin (1:1,024) was also added to bacteria grown in NBD to estimate its possible activating effect on yersiniabactin synthesis genes. Total-cell lysates were analyzed by SDS-PAGE (7.5% gel) (Fig. 8). Neither pesticin nor the supernatant had any significant effect on the expression of both HMW proteins in the wild-type strain. However, in contrast to pesticin, the yersiniabactin-containing supernatant restored expression of HMWP2 but not of HMWP1 in the mutant *irp1* strain. The same results were obtained by the addition of purified yersiniabactin to the iron-deficient media (Fig. 7).

The effect of yersiniabactin on *fyuA* expression was examined in the *irp1* mutant carrying a plasmid with *fyuA* translationally fused to the *gfp* reporter gene. The induction of FyuA-GFP leads to bright green fluorescent wild-type yersiniae in iron-deficient NBD medium. In contrast, the fluorescence of mutant WA-CS *irp1*::Kan^r was much weaker. Fluorescent microscopy as well as FACSscan analysis revealed no increase in fluorescence when a sublethal dose of pesticin was supplied (data not shown), suggesting that pesticin does not act as an inducer of its receptor. In contrast, addition of purified yersiniabactin to the *irp1* mutant results in a higher level of green fluorescence than in the wild type (Fig. 9). Addition of another siderophore (desferrioxamine B) or of a molecule structurally related to yersiniabactin (pyochelin) does not lead to an increase of the *fyuA* expression, indicating a specific induction of receptor expression by its own siderophore.

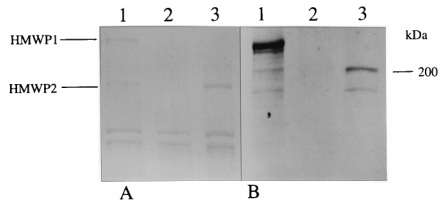

FIG. 7. SDS-PAGE (7.5% gel) (A) and corresponding immunoblot (B) with cell lysates of WA-CS (lane 1), WA-CS *irp1*::Kan^r (lane 2), and WA-CS *irp1*::Kan^r with an addition of purified yersiniabactin (lane 3). The strains were grown under iron starvation in NBD medium. Western blotting was performed with HMWP polyclonal antibodies kindly provided from Elisabeth Carniel.

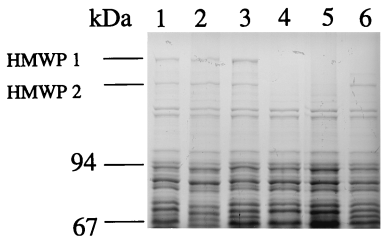

FIG. 8. Effects of pesticin and yersiniabactin on expression of the HMWPs. SDS-PAGE (7.5% gel) of total-cell proteins of iron-starved strains WA-CS and WA-CS *irp1*::Kan^r with addition of pesticin and culture supernatant containing yersiniabactin. Lane 1, WA-CS in NBD medium; lane 2, WA-CS in NBD medium with pesticin (1:1,024); lane 3, WA-CS in NBD medium with yersiniabactin supernatant (1:50); lane 4, WA-CS *irp1*::Kan^r in NBD medium; lane 5, WA-CS *irp1*::Kan^r in NBD medium with pesticin; lane 6, WA-CS *irp1*::Kan^r in NBD medium with yersiniabactin supernatant.

No siderophore synthesis could be detected in the *irp1* mutant, but the cells were still able to grow in iron-deficient medium. The mutant strain grows in NBD medium with a final α-α-dipyridyl concentration of 400 μg/ml, indicating the presence of another iron uptake system in addition to the yersiniabactin system.

DISCUSSION

Since the rediscovery of siderophore production by highly pathogenic *Yersinia* species in 1987, it has been generally appreciated that this high-affinity ferric iron uptake system significantly contributes to virulence of yersiniae (31). Recently, the chemical structure of yersiniabactin has been determined (18). There was an indication that the putative genes for biosynthesis of yersiniabactin reside within the HPI on the chromosome (28). The objectives of this study were to demonstrate that the *irp1* and *irp2* genes, encoding HMWP1 and HMWP2, are involved in yersiniabactin biosynthesis and to characterize the *irp* operon. Two genes, *irp2*, encoding iron-repressible HMWP2, which is proposed to be involved in nonribosomal protein synthesis, and *fyuA*, encoding yersiniabactin/pesticin FyuA receptor, represent the yersiniabactin biosynthetic cluster. A small *araC*-like *ybt*A gene seems to be a positive regulator for *irp* gene expression (22) and precedes the *irp* operon.

In this study, we analyzed the whole *irp* operon and identified four additional ORFs on a 13-kb DNA fragment between the *irp2* and *fyuA* genes. The largest one, located immediately downstream *irp2*, comprises 9.5 kb with the capacity to code for a 384-kDa polypeptide. The theoretical molecular mass of the polypeptide encoded by that ORF is higher than expected from the results of the SDS-PAGE (240 kDa). This can be due to the complex secondary structure of this extremely large protein as well as to its possible atypical migration in denaturing conditions. The insertional inactivation of *irp1* by allelic exchange leads to loss of HMWP1 and HMWP2 in SDS-PAGE (Fig. 6).

Inactivation of *irp1* or *irp2* resulted in the loss of siderophore

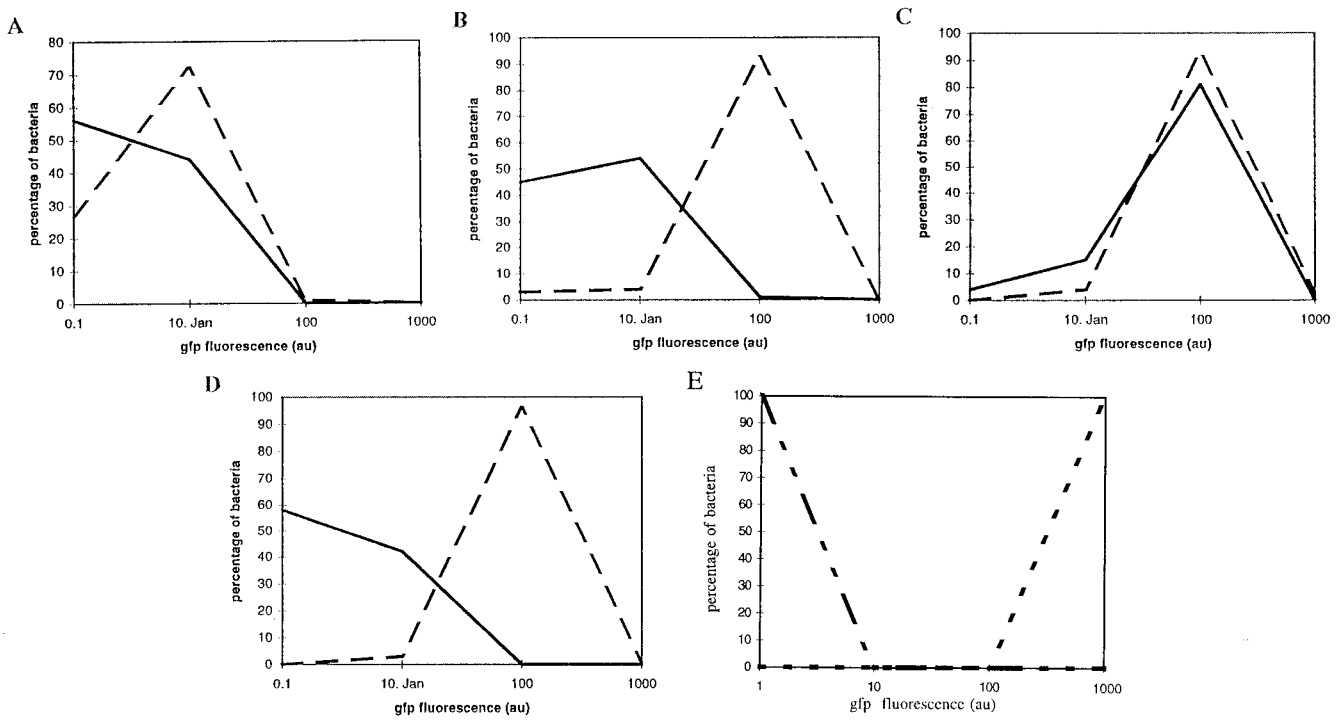

FIG. 9. Fluorescence of FyuA-GFP in *Y. enterocolitica* WA-CS (- -) and WA-CS *irp1*::Kan^r (—) in NB medium (A), NBD medium (B), NBD medium plus purified yersiniabactin (C), and NBD medium plus desferrioxamine B (D). (E) Positive (WA-CS[pGFP mut 3]; ▪ ▪) and negative (WA-CS; ▪ ═ ▪) controls. Au, arbitrary units.

production as monitored on CAS agar (Fig. 5). Thus, *irp1* and *irp2* are involved in yersiniabactin biosynthesis.

HMWP1 shares a unique motif with the polyketide synthases. The β-ketoacyl-ACP synthase active site is highly conserved among the three multifunctional polypeptides of the rapamycin-producing polyketide synthases of *S. hygroscopicus* as well as in the polyketide synthase of *S. antibioticus* (1). Polyketide synthases are involved in the synthesis of a large and highly diverse group of heterocyclic compounds including antibiotics, antitumor compounds, and heterocyclic immunosuppressants. Polyketide metabolites are produced by successive condensation of simple acid units as propionate and acetate. Prolonging of the acid chain is catalyzed by the polyketide synthases (17). The similarity implies that HMWP1 could be involved in the synthesis of a siderophore or of an antibiotic.

The last 6.3 kb of *irp1* have no significant similarity to any known sequence besides a 52.9% identity over 1,481 bp (bp 5716 to 7132) to *irp2*. HMWP2 is known to be homologous to AngR (involved in the anguibactin biosynthesis of *V. anguillarum*), and it was predicted to direct nonribosomal synthesis of small molecules involved in the nonribosomal synthesis of antibiotics or siderophores (28).

Two other defined ORFs, *irp4* and *irp5*, have pronounced identity to a thioesterase-like protein from anguibactin biosynthetic gene cluster of *V. anguillarum* (20) and EntE (2,3-dihydroxybenzoic acid-activating enzyme) from *E. coli* (57), respectively. *irp4* and *irp5* are nearly identical to the *ybtT* and *ybtE* genes described as a part of a siderophore biosynthetic region in *Y. pestis* (3). Insertional inactivation of the *ybtE* gene yielded mutants unable to grow in iron-deficient medium at 37°C. A *ybtE* mutant could be cross-fed by a culture supernatant from a wild-type strain. It is reasonable to propose that yersiniabactin or a siderophore with a similar structure can represent this siderophore in *Y. pestis*. Taking into account structural similarities of yersiniabactin and anguibactin siderophores (Fig. 10), one can assume that *irp2* to *irp5* represent a yersiniabactin biosynthetic gene cluster with the following organization: *irp2-irp1-irp3-irp4-irp5*. An additional gene located between *irp2* and *irp1* as proposed for *Y. pestis* (3) could not be identified.

Insertional inactivation of the *irp1* and *irp2* yersiniabactin biosynthetic genes results not only in elimination of the corresponding polypeptide bands but also in downregulation of the other proteins involved in yersiniabactin synthesis and binding;

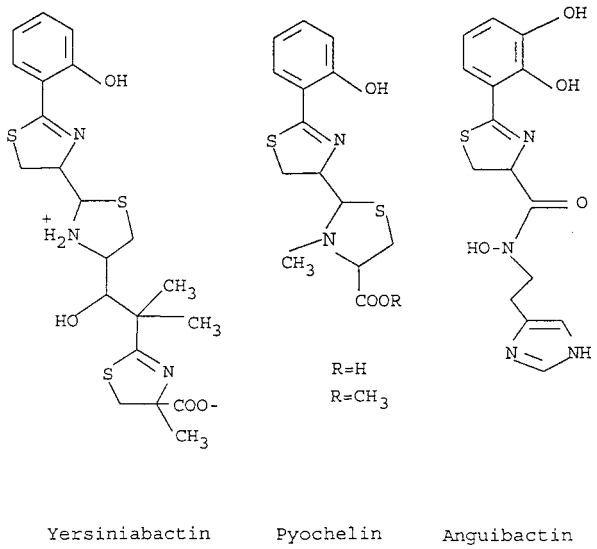

FIG. 10. Chemical structures of yersiniabactin, pyochelin, and anguibactin.

namely, *irp1* inactivation was followed by considerable reduction of HMWP2 and FyuA proteins (Fig. 6 and 9). Nonsense mutation in the *fyuA* gene resulted in downregulation of the *irp1* and *irp2* genes. It was expected that inactivation of the *irp2* gene, being the first gene in the polycistronic operon, would lead to a reduction of the *irp1* gene product since *irp1* is devoid of promoter/operator sequences. The unexpected reduction of HMWP2 and FyuA production as a result of *irp1* inactivation suggests that the yersiniabactin biosynthetic operon is subjected to autoregulation by its product, the siderophore. Indeed, the addition of yersiniabactin to the siderophore-negative mutant WA-CS *irp1*::Kan^r upregulates the production of HMWP2 (Fig. 7 and 8) and its receptor, as demonstrated by the translational *fyuA-gfp* fusion (Fig. 9). Consistent with these data, *Y. pestis* supernatant was also found to influence the expression of the *ybt* (yersiniabactin biosynthetic genes in *Y. pestis*)-encoded proteins (3).

Exogenous siderophore desferrioxamine B taken up by yersiniae through the FoxA receptor (2) did not induce *fyuA*. Thus, the yersiniabactin molecule specifically induces *fyuA* expression, while sublethal doses of neither pesticin, desferrioxamin B, nor the structurally related molecule pyochelin could serve as a signal for induction of the *irp* operon.

Siderophore-dependent expression of the cognate receptors was demonstrated for the iron dicitrate system in *E. coli* (61) and for pyoverdine, pyochelin, and enterobactin receptors in *Pseudomonas aeruginosa* (16, 34, 59). Moreover, phenolate siderophore pyochelin shows high structural similarity with yersiniabactin (Fig. 10). Several other features are common between yersiniabactin and pyochelin receptors. The presence of pyochelin, which exhibits a low affinity for iron in vitro, has been correlated with increased virulence and in vivo growth (13). The pyochelin receptor gene *fptA* is positively regulated by a *pchR* product, an AraC-type transcriptional regulator (34). On the basis of the similarity of *ybtA* to the *araC*-type regulators, it was proposed that the YbtA activator requires ferric yersiniabactin to interact with the palindrome sequences upstream *psn* (designation of the yersiniabactin receptor in *Y. pestis* with 99% similarity to *fyuA*) and *irp2* for the maximum induction of these genes (22). Thus, the absence of yersiniabactin results in low expression of *irp2* and *fyuA* genes in the *irp1* mutant due to the inability of YbtA to form a complex with yersiniabactin, to bind to the palindrome sequences, and therefore to activate the yersiniabactin operon. Due to the lack of sequencing data of the pyochelin biosynthetic cluster, it is not possible to demonstrate the relationship of these two siderophore systems on the molecular level. Nevertheless, these three siderophore systems, yersiniabactin, pyochelin, and anguibactin, have a high degree of similarity in structure and function (Fig. 10).

Although it exhibited no siderophore production, the mutant WA-CS *irp1*::Kan^r was still able to grow in iron-deficient medium. This indicates that at least one efficient iron uptake system is present in *Y. enterocolitica* in addition to the yersiniabactin system. The TonB-independent *yfu* system discovered recently in *Y. enterocolitica* (49) may be a candidate for such a system.

irp2 and *fyuA* genes present on the HPI were shown to be highly conserved (45). The same is true to the *irp1* gene start and end portions, which were shown to be identical or highly similar in all three highly pathogenic species, *Y. enterocolitica*, *Y. pseudotuberculosis* O1, and *Y. pestis*. The *irp4* and *irp5* genes were also found to be nearly identical to the corresponding genes identified in *Y. pestis* KIM. Thus, *ybtA-fyuA* genes comprise a highly conserved gene cluster (HPI) present in highly pathogenic yersiniae. A high G+C content and a codon usage different from that in *Yersinia* housekeeping genes were found in all genes constituting the HPI. Therefore, a horizontal transfer may be responsible for the dissemination of the yersiniabactin biosynthetic operon in *Yersinia*. Moreover, recent studies have demonstrated that conserved HPI is widely distributed among representatives of certain pathotypes of *E. coli* (50). Such a wide dissemination of HPI and its impact on the virulence of *Yersinia* implies an important, possible multifunctional role of yersiniabactin system in vivo and the availability of an efficient mechanism for its genetic transfer.

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