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Analysis of the Pesticin Receptor from *Yersinia pestis*: Role in Iron-Deficient Growth and Possible Regulation by Its Siderophore

JACQUELINE D. FETHERSTON, JAMES W. LILLARD, JR., AND ROBERT D. PERRY*

Department of Microbiology and Immunology, University of Kentucky, Lexington, Kentucky 40536-0084

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We have sequenced a region from the *pgm* locus of *Yersinia pestis* KIM6+ that confers sensitivity to the bacteriocin pesticin to certain strains of *Escherichia coli* and *Y. pestis*. The *Y. pestis* sequence is 98% identical to the pesticin receptor from *Yersinia enterocolitica* and is homologous to other TonB-dependent outer membrane proteins. *Y. pestis* strains with an in-frame deletion in the pesticin receptor gene (*psn*) were pesticin resistant and no longer expressed a group of iron-regulated outer membrane proteins, IrpB to IrpD. In addition, this strain as well as a *Y. pestis* strain with a mutation constructed in the gene (*irp2*) encoding the 190-kDa iron-regulated protein HMWP2 could not grow at 37°C in a defined, iron-deficient medium. However, the *irp2* mutant but not the *psn* mutant could be cross-fed by supernatants from various *Yersinia* cultures grown under iron-deficient conditions. An analysis of the proteins synthesized by the *irp2* mutant suggests that HMWP2 may be indirectly required for maximal expression of the pesticin receptor. HMWP2 likely participates in synthesis of a siderophore which may induce expression of the receptor for pesticin and the siderophore.

The studies of Jackson and Burrows in the 1950s were the first indication that an iron transport system, functioning at 37°C, is linked to the pigmentation phenotype (Pgm^+) of *Yersinia pestis*, the causative agent of plague (35). They showed that virulence could be restored to nonpigmented (Pgm^-) strains of *Y. pestis* by simultaneously injecting iron-containing compounds intraperitoneally into mice. Sikkema and Brubaker (57) demonstrated that Pgm^+ strains of *Y. pestis* could maintain growth at 37°C in a chemically defined, iron-chelated medium whereas most Pgm^- organisms could not. Both Pgm^+ and Pgm^- strains readily grew in this medium at 26°C. The ability to grow in iron-deficient media at 37°C correlated with sensitivity to the bacteriocin pesticin and with the expression of a group of iron-regulated outer membrane proteins, IrpB to IrpE, that have similar molecular weights but slightly different isoelectric points. These results suggested that the pesticin receptor corresponds to one or more of the IrpB to IrpE proteins and is involved in the assimilation of iron at 37°C (58).

In *Yersinia enterocolitica*, pesticin sensitivity is associated with the expression of a 65-kDa iron-regulated outer membrane protein (Irp65 or FyuA) which is also the receptor for the siderophore yersiniabactin (28, 30, 52). *Y. enterocolitica* and *Yersinia pseudotuberculosis* strains that expressed Irp65 and had siderophore activity were lethal by intravenous injection in mice (30). We have shown that in *Y. pestis*, pesticin sensitivity and a number of other traits associated with the Pgm^+ phenotype are linked to a 102-kb region of chromosomal DNA termed the *pgm* locus (19, 20, 48). An ~5.9-kb fragment from this region restores pesticin sensitivity to *Y. pestis* strains which have undergone a spontaneous deletion of the entire 102-kb locus, as well as to certain strains of *Escherichia coli* (19). In *E. coli* minicells and an in vitro transcription-translation system, the cloned DNA directs the synthesis of a 68-kDa protein which appears to be derived from a 72-kDa precursor. This same clone restores limited expression of the IrpB to IrpD proteins as determined by ^{125}I surface-labeling techniques (19). Of the IrpB to IrpE proteins, only IrpB to IrpD can be surface labeled with ^{125}I (46).

In addition to pesticin sensitivity, an ~40-kb fragment from the *pgm* locus restored the expression of two high-molecular-weight iron-regulated proteins, approximately 240 and 190 kDa in size, to a Pgm^- strain of *Y. pestis* (19). These proteins probably correspond to the high-molecular-weight iron-regulated proteins HMWP1 and HMWP2, first identified by Carniel et al. in *Y. enterocolitica* and shown to be expressed by pathogenic strains of yersiniae under iron-deficient conditions (8). The gene, *irp2*, that encodes the 190-kDa HMWP2 is absent from weakly pathogenic and avirulent strains (10). In *Y. pestis* KIM6+, the *irp2* gene is located within the *pgm* locus, upstream of the sequences that restore pesticin sensitivity (19). Mutations in the *irp2* gene of *Y. pseudotuberculosis* eliminate the expression of both the 190- and 240-kDa HMWPs and result in a strain with reduced virulence which can be partially restored by administering iron (9). Nucleotide sequence analysis of *irp2* revealed that its product shares homology with a group of proteins involved in the nonribosomal synthesis of peptide bonds, in particular those proteins required for the synthesis of certain antibiotics and siderophores (27). Recent studies have indicated that *Y. enterocolitica* does produce a siderophore, termed yersiniabactin (28) or yersiniophore (11). Chambers and Sokol found that the production of yersiniophore correlated with the presence of five iron-regulated proteins, 240, 190, 80, 71, and 67 kDa in size (11). Collectively, these results suggest that the 190-kDa polypeptide and probably additional proteins are involved in the synthesis of the siderophore yersiniabactin, whose uptake is mediated by the pesticin receptor (27, 30). Whether or not *Y. pestis* produces a siderophore has been controversial (47, 69). While it is known that *Y. pestis* cells synthesize HMWP1, HMWP2, and a pesticin receptor, a role for these proteins in iron transport has not been definitively demonstrated.

We have cloned and sequenced the pesticin receptor gene (*psn* [38]) from *Y. pestis* KIM6+. The *psn* gene is 98% identical

* Corresponding author. Phone: (606) 323-6341. Fax: (606) 257-8994. Electronic mail address: rdp@seqanal.mi.uky.edu.

TABLE 1. Bacterial strains and plasmids used[a]

Strain or plasmid	Relevant characteristics	Reference(s)
Y. pestis		
KIM5	Δ*pgm* Pst^r Lcr^+	19, 23
KIM6+	*hms*$^+$ *psn*$^+$ *irp1*$^+$ *irp2*$^+$ Pst^+	20, 57
KIM6	Δ*pgm* Pst^+	20, 57
KIM10+	*hms*$^+$ *psn*$^+$ *irp1*$^+$ *irp2*$^+$ Pst^s	48
KIM10	Δ*pgm* Pst^r	48
KIM6-2030+	*hms*$^+$ *psn*$^+$ *irp1*$^+$ *irp2*$^+$ *fur*::*kan-9* Pst^+ Km^r	60
KIM6-2030	Δ*pgm* *fur*::*kan-9* Pst^+ Km^r	60
KIM10-2030(pYH02)+	*hms*$^+$ *psn*$^+$ *irp1*$^+$ *irp2*$^+$ *fur*::*kan-9* *Pst*^{s} Ap^r Km^r	60
KIM6-2045	*hms*$^+$ *psn*::pPSN15 *irp1*$^+$ *irp2*$^+$ Pst^+ Ap^r	This study
KIM6-2045.1	*hms*$^+$ Δ*psn* *irp1*$^+$ *irp2*$^+$ Pst^+ Ap^s	This study
KIM10-2045.2	*hms*$^+$ *psn*::pPSN15 *irp1*$^+$ *irp2*$^+$ Pst^s Ap^r	This study
KIM10-2045.5	*hms*$^+$ Δ*psn* *irp1*$^+$ *irp2*$^+$ Pst^r Ap^s	This study
KIM6-2046	*hms*$^+$ *psn*$^+$ *irp2*::pCIRP498.8 Pst^+ Ap^r Km^r HMWP1$^-$	This study
KIM6-2046.1	*hms*$^+$ *psn*$^+$ *irp2*::*kan* Pst^+ Ap^s Km^r HMWP1$^-$	This study
Y. pseudotuberculosis PB1/0	*psn*$^+$ *irp2*$^+$ Pst^s	20, 67
Y. enterocolitica WA-LOX	*psn*$^+$ *irp2*$^+$ Pst^s	20, 67
E. coli		
DH5α	Cloning strain, Pst^r	3
K-12	Generic strain, Pst^r	47
SY327(λ*pir*)	Strain for propagating plasmids with R6K origins	42
XL1-Blue	Cloning and M13 propagating strain	54
Plasmids		
pSDR498	41-kb *Bam*HI fragment from *Y. pestis* KIM6+ *pgm* locus into pHC79, Ap^r	20
pSDR498.4	23-kb *Bgl*II fragment from pSDR498 into pBGL2, Ap^r	20
pSDR498.8	19.2-kb *Bam*HI-*Sal*I fragment from pSDR498 into pBGL2, Ap^r	19
pKAN498.8	Insertion of 1.7-kb *Bam*HI fragment containing the Km^r region from the Tn*10* derivative mini-*kan* (70) into *Bgl*II site of pSDR498.8, Ap^r Km^r	This study
pCIRP498.8	11.2-kb *Sal*I fragment from pKAN498.8 into pCVD442, Ap^r Km^r	This study
pIRP2	9.5-kb *Sal*I fragment encoding *irp2* from pSDR498.8 into pBGL2, Ap^r	This study
pPSN2	6.0-kb *Bgl*II-*Sal*I fragment from pSDR498.4 into pHC79, Ap^r	19
pPSN4	4.2-kb *Pst*I-*Bam*HI fragment from pPSN2 into pUC18, Ap^r	This study
pPSN13	4.2-kb *Hin*dIII-*Sal*I fragment from pPSN4 into pBGL2, Ap^r	This study
pPSN14	Deletion of 447-bp *Eco*RV fragment from pPSN4, Ap^r	This study
pPSN15	2.65-kb *Hin*cII fragment from pPSN14 into pCVD442, Ap^r	This study
pPSN18	3.1-kb *Hin*cII fragment from pPSN4 into pUC18, Ap^r	This study
pUC18	2.7-kb cloning vector, Ap^r	54
pHC79	6.4-kb cosmid cloning vector, Ap^r Tc^r	32
pBGL2	4.8-kb cloning vector, Ap^r Tc^r	48
pCVD442	6.2-kb suicide vector, Ap^r *sacB*$^+$	17

[a] *Y. pestis* strains designated with a "+" possess an intact 102-kb *pgm* locus that includes the hemin storage locus (*hms*), *psn*, and *irp2* genes. Those without a "+" designation either have deleted the entire region (Δ*pgm*) or contain a mutation in the *pgm* locus. Pst^+ indicates strains carrying the plasmid encoding pesticin and its immunity protein. Bacterial strains missing this plasmid are either sensitive (Pst^s) or resistant (Pst^r) to the action of pesticin. An Lcr^+ phenotype indicates the ability to expression calcium- and temperature-regulated virulence genes that are part of the low-calcium response regulon encoded on an ~75-kb plasmid. Of the *Yersinia* strains used in this study, only KIM5 is Lcr^+. Km^r, Ap^r, and Tc^r, resistance to kanamycin, ampicillin, and tetracycline.

at both the nucleotide and predicted amino acid sequence levels to the *Y. enterocolitica* pesticin receptor, FyuA (52), and shows homology to other TonB-dependent siderophore receptors. An engineered *Y. pestis* strain containing an in-frame deletion in *psn* was no longer sensitive to pesticin and was unable to grow on iron-deficient media at 37°C. Similarly, a *Y. pestis* strain containing a polar mutation in *irp2* could not grow at 37°C under iron-deficient conditions. Supernatants from various *Yersinia* cultures grown under iron-deficient conditions could stimulate the growth of the *irp2* but not the *psn* mutant. Finally, an analysis of the proteins synthesized by the *irp2* mutant strains suggests that HMWP2 may be indirectly required for efficient expression of the pesticin receptor.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The plasmids and bacterial strains used in this study are listed in Table 1. The hemin storage (Hms) (19, 48) phenotype of all *Y. pestis* strains was determined on Congo red plates (64). Pesticin sensitivity of bacterial strains was determined as previously described (7, 19). *Yersinia* strains were grown in either heart infusion broth (HIB) or the defined medium, PMH, rendered iron-deficient by extraction with Chelex 100 (61). Both Pgm^+ and Pgm^- cells of *Y. pestis* were able to grow at 37°C on iron-deficient PMH solidified with Noble agar. PMH plates containing 0.5 mM $NaCO_3$, 0.01 mM $MnCl_2$, and 4 mM $CaCl_2$ (PMH-S) supported the growth of Pgm^+ cells at 37°C but not Pgm^- cells. Since $CaCl_2$ precipitates iron (13), this treatment presumably lowered the iron concentration in the media below the level required for the 37°C growth of Pgm^- cells. Both Pgm^+ and Pgm^- strains grew on PMH-S at 26°C. *E. coli* strains were grown in Luria broth, 2× TY medium (3), or Terrific Broth (65). M13 bacteriophage were plated in H top agar onto H plates (3). Where appropriate, media contained ampicillin or kanamycin at 50 μg/ml or tetracycline at 12.5 μg/ml. Sensitivity to the bacteriocin pesticin was tested as previously described (19).

Nucleotide sequencing and analysis. Restriction fragments from the 3.1-kb *Hin*cII fragment of pPSN18 were subcloned into M13mp18 or -19 and transformed into *E. coli* XL1-Blue cells. Bacteriophage were precipitated from infected cell culture supernatants by the addition of polyethylene glycol (54). Single-stranded DNA, isolated from the phage, was sequenced by the dideoxynucleotide chain termination method (55) using [^{35}S]dATP (Amersham Corp.) and Sequenase version 2.0 (U.S. Biochemical Corp.). Samples were electrophoresed at 75 W on 6% polyacrylamide gels containing 8.3 M urea and cast in Tris-borate-EDTA buffer (54). Dried gels were exposed to Kodak BioMax MR film at room temperature. Synthetic oligonucleotide primers (Integrated DNA Technologies, Inc.) were used to extend the sequence. PC/Gene was used to identify potential promoter elements and signal peptides, while the IntelliGe-

netics suite series of programs was used for other DNA analyses. Homology searches were performed by using BLAST (1). Multiple sequence alignments were done with Pileup from version 7.3-VMS of the Genetics Computer Group software package from the University of Wisconsin (16).

Recombinant DNA techniques. Plasmids were purified by alkaline lysis (4) or polyethylene glycol precipitation (33). A standard $CaCl_2$ procedure was used to introduce plasmids into *E. coli* cells (54). *Y. pestis* cells were transformed by electroporation, using a slight modification of the protocol of Conchas and Carniel (12). Briefly, an overnight culture was diluted to 0.2 OD_{620} (optical density at 620 nm) units and grown at 37°C to an OD_{620} of 0.8. The cells were pelleted, washed once in ice-cold sterile water and once in ice-cold sterile 10% glycerol, and then resuspended in 10% glycerol to a final concentration of 50 OD_{620} units/ml. Five hundred nanograms of plasmid DNA was added to 20 μl of cells and incubated for 20 min on ice. Cells were electroporated in an ice slurry at 400 V, using a Bethesda Research Laboratories Cell-Porator set at low ohms and 330 μF with a booster setting of 4 kΩ. This typically delivered a field strength of 12.6 kV/cm (1.9-kV discharge with an electrode gap of 0.15 cm). The cells were removed from the chamber with 1.0 ml of HIB and incubated for 1 h at 37°C before plating on the appropriate media.

For hybridization to oligonucleotide probes, genomic DNA was isolated as previously described (20), digested with *Eco*RI, separated on a 0.7% agarose gel, and transferred to nitrocellulose (59). Blots were prehybridized at 55°C in 6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) (54)–5× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml and then hybridized overnight at 55°C in 6× SSPE–1× Denhardt's solution–0.1% SDS–100 μg of yeast tRNA per ml to 4 ng of a synthetic oligonucleotide, AAATAAACCCGTTTCGGGTAGC (Integrated DNA Technologies, Inc.), that was end labeled with terminal deoxynucleotidyltransferase to a specific activity of 5×10^9 cpm/μg. The blots were washed in 6× SSC, twice for 15 min each at room temperature and once for 15 min at 42°C.

Generating *psn* and *irp2* mutant strains. An in-frame deletion was made in the pesticin receptor gene by deleting a 447-bp *Eco*RV fragment (Fig. 1) from pPSN4 to generate pPSN14. The gene encoding the 190-kDa iron-regulated protein, HMWP2, was disrupted by inserting a kanamycin resistance gene cassette into the unique *Bgl*II site in pSDR498.8, creating pKAN498.8 (Table 1). DNA sequence around this *Bgl*II site (GenBank accession number U19396) shows near identity with the *irp2* gene of *Y. enterocolitica* (27). The mutated genes were then cloned into the suicide vector pCVD442 (17), generating pPSN15 and pCIRP498.8, respectively. The vector contains an R6K origin of replication that requires the *pir* gene product (36) and the *sacB* gene from *Bacillus subtilis*, which renders certain bacterial strains sensitive to sucrose (21). The plasmids were electroporated into *Y. pestis* KIM6+; pPSN15 was also transformed into KIM10+. Merodiploids were selected for growth on plates containing ampicillin for strains transformed with pPSN15 or ampicillin and kanamycin for strains containing pCIRP498.8. Genomic DNA from selected colonies was checked by Southern blot analysis to ensure that the plasmids had integrated properly. The cointegrant strains KIM6-2046 (*irp2*::pCIRP498.8) and KIM6-2045 (*psn*::pPSN15) as well as KIM10-2045.2 (*psn*::pPSN15) were grown overnight in HIB with and without kanamycin respectively. An aliquot, 0.001 OD_{620} units, of the overnight cultures was spread on Congo red plates containing 5% sucrose or sucrose and kanamycin for the KIM6-2046 culture. Genomic DNA from the Suc^r colonies was screened by Southern blot analysis to identify those which had exchanged the mutant gene for the wild type.

Protein analyses. In vitro transcription-translation assays were performed by using an *E. coli* S30 extract system and the conditions recommended by the supplier (Promega Corp.). Acetone-precipitated products were resuspended in sample buffer and electrophoresed on 12% polyacrylamide gels containing SDS (39). The gels were dried and exposed to Kodak X-Omat AR5 film at −20°C.

Cellular proteins of *Y. pestis* strains, acclimated to growth under iron-deficient or iron-sufficient conditions, were labeled with ^{35}S-amino acids for 1 h as previously described (19). Equal amounts of trichloroacetic acid-precipitable counts were electrophoresed on polyacrylamide gels in the presence of SDS and analyzed by autoradiography. *Y. pestis* surface proteins were labeled with ^{125}I as previously described (62). The proteins were analyzed by two-dimensional gel electrophoresis, using isoelectric focusing in the first dimension followed by electrophoresis on 12% polyacrylamide gels (45).

Feeding studies. Culture supernatants were obtained from cells inoculated into deferrated PMH and grown for a total of six to nine generations at 37°C. The cells were pelleted by centrifugation, and the supernatant was filtered through a 0.2-μm-pore-size filter. For growth responses, a PMH-S plate was overlayered with 0.04 OD_{620} units of cells grown in deferrated PMH as described above, and approximately 25 μl of the filtered supernatant was added to wells in the plate. The plates were incubated at 37°C. For analysis of protein synthesis responses, cells acclimated to iron-deficient growth were transferred to cultures containing 50% fresh deferrated PMH and 50% filtered supernatant. Cultures were incubated at 37°C for 1.5 h prior to labeling with ^{35}S-amino acids. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U09530.

RESULTS

Sequencing and analysis of the *psn* gene. The nucleotide sequence of an ~2.3-kb region of the *Y. pestis* KIM6+ *pgm* locus that conferred pesticin sensitivity to DH5α, an *E. coli* K-12 derivative that is normally resistant (18, 19), is shown in Fig. 1. An open reading frame of 2,019 bp was found within this sequence beginning at nucleotide 161 and terminating at 2182, which would generate a 673-amino-acid protein with a predicted molecular weight of 73,786. A putative 22-amino-acid signal peptide is located at the amino terminus and upon removal would yield a mature product with a molecular weight of 71,476. The predicted protein sequence has features found in other outer membrane proteins, including a β-sheet structure at the carboxy terminus and a terminal phenylalanine residue (63). In addition, the *psn* gene product (IrpC) contains regions commonly present in TonB-dependent proteins (Fig. 1 and reference 41).

The *psn* gene from *Y. pestis* shows the strongest homology to the gene encoding *Y. enterocolitica* pesticin receptor (FyuA [52]), being 98% identical at both the nucleotide and deduced amino acid sequence levels. Of the seven scattered amino acid differences, five are conservative changes. IrpC also has about 40 to 50% similarity with other siderophore receptors as well as IrgA and CirA (Fig. 2). The strongest homology was found in the four domains that are common to TonB-dependent proteins (41). Outside of these domains there were scattered regions of homology. In some areas, amino acids 417 to 435, for example, the pesticin receptor resembled the *Pseudomonas* siderophore receptors PupA, FpvA, and FptA, while in other regions (amino acids 203 to 220) it was more closely related to CirA, IrgA, and FepA.

An inverted repeat, present at the 3′ end of *psn* (Fig. 1), may function in terminating transcription. Potential −35 and −10 regions as well as a ribosome binding site are indicated in Fig. 1. Overlapping the putative −10 region is an inverted repeat which is homologous to the *E. coli* consensus Fur-binding site (FBS) (6). The Fur protein usually acts as a negative transcriptional regulator using iron as a cofactor (44). In *Y. enterocolitica* and *Y. pestis*, pesticin sensitivity is increased by mutations in *fur* (30, 60, 62). Upstream of the potential FBS is another inverted repeat (Fig. 1) which is nearly identical in both composition and relative position to a sequence found upstream of the *irp2* gene in *Y. enterocolitica* (27). To determine if this sequence is present elsewhere in the genome, an oligonucleotide corresponding to the right half of the stem structure was used to probe DNA from various strains. A clone, pSDR498, from the *pgm* locus of *Y. pestis* that restores HMWP1 and HMWP2 expression as well as pesticin sensitivity (19) contained two *Eco*RI fragments, 14.8 and 3.3 kb, that hybridized to the oligonucleotide (Fig. 3). These same two fragments were present in the *Y. pestis* Pgm^+ strains KIM6+ and KIM10+. Pgm^- strains KIM5, KIM6, and KIM10 have deleted the entire 102-kb *pgm* locus and show only faint hybridization to a single fragment of ~15 kb. These results suggest that in *Y. pestis*, sequences that are highly homologous to this inverted repeat are present only in the *pgm* locus. *Y. pseudotuberculosis* PB1/0 genomic DNA contained two bands, similar in size to the *Y. pestis* fragments that hybridized strongly to the probe, while one of the fragments was smaller (~10 kb) in *Y. enterocolitica* WA-LOX DNA. In addition, faint hybridization was detected to a third fragment (~6 kb) in *Y. enterocolitica*. No specific hybridization was apparent to DNA from *E. coli* K-12. Thus, there appear to be at least two very homologous copies of this portion of the inverted repeat sequence in the *Y. pestis* genome, presumably upstream of *irp2* and *psn*. Additional,

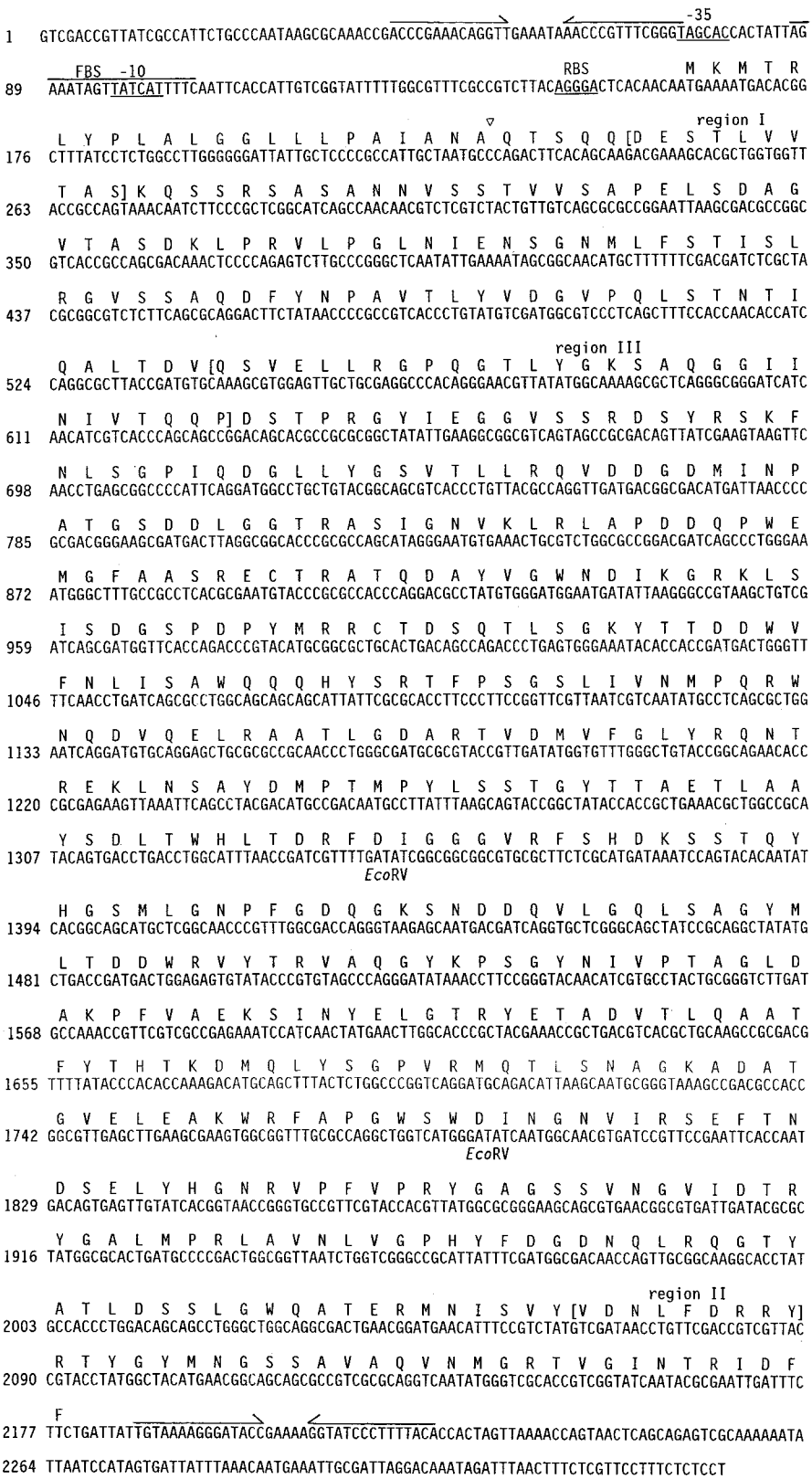

FIG. 1. Nucleotide and predicted amino acid sequences of the pesticin receptor from *Y. pestis*. The complete nucleotide sequence of the *psn* gene from *Y. pestis* KIM6+ is shown. Potential −35 and −10 regions as suggested by Rakin et al. (52) as well as a possible ribosome binding site (RBS) are underlined. A region showing homology to the Fur-binding consensus sequence (FBS) is overlined. Inverted repeat structures near the beginning and end of the sequence are overlined by half arrows. The *Eco*RV sites used to generate an in-frame deletion are also indicated. Regions within the predicted amino acid sequence that are found in other TonB-dependent proteins are enclosed in brackets, and the potential signal peptidase cleavage site is indicated by ▽.

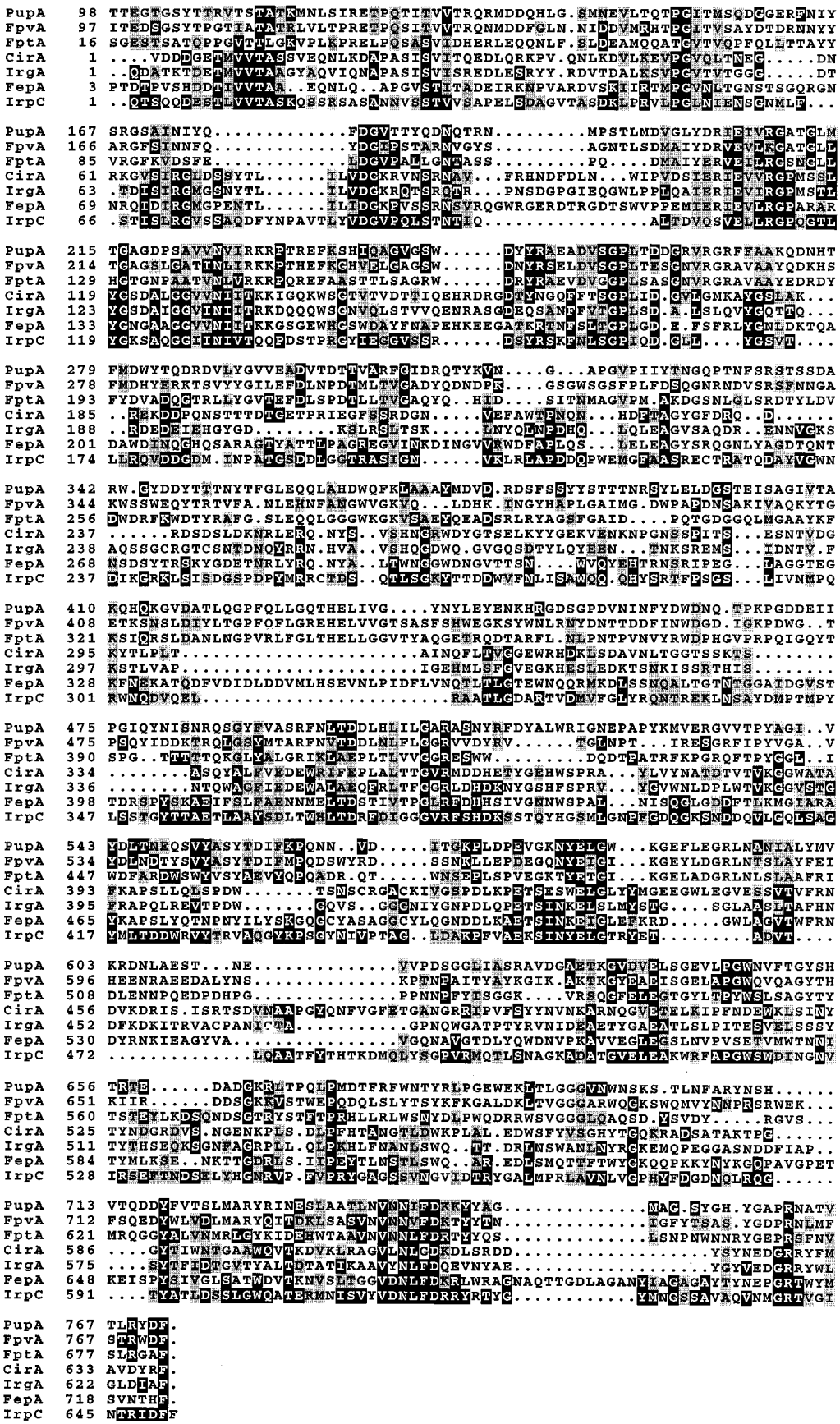

FIG. 2. Alignment of the predicted amino acid sequence of the pesticin receptor (IrpC) with sequences of PupA (5), FpvA (49), FptA (2), CirA (43), IrgA (24), and FepA (41). Relative to IrpC, identical residues appear as white letters within a black background, while conservative amino acid changes are shown as black letters on a gray background.

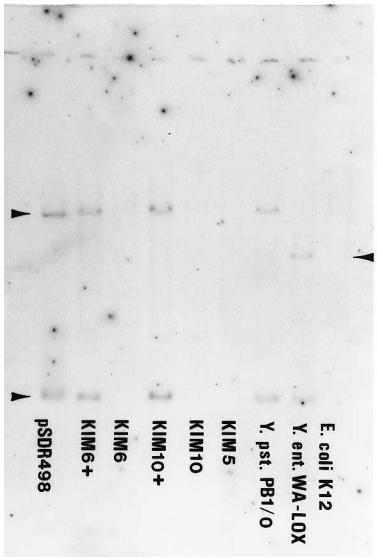

FIG. 3. Southern blot probed with an oligonucleotide from the *psn* promoter region. *Eco*RI-digested DNA from the indicated *Y. pestis* strains (KIM6+, KIM6, KIM10+, KIM10, and KIM5), *Y. pseudotuberculosis* (Y. pst. PB1/0), *Y. enterocolitica* (Y. ent. WA-LOX), and *E. coli* K-12 were hybridized to a 22-mer containing sequences from the inverted repeat located within the promoter region of *psn*. Plasmid DNA (pSDR498), which contains sequences encoding HMWP2 and the pesticin receptor, was included as a control. Arrowheads point to strongly hybridizing bands.

weakly related sequences may be present outside the *pgm* locus.

Construction and characterization of a *Y. pestis* pesticin-resistant mutant. We constructed an in-frame deletion in the *Y. pestis* pesticin receptor gene by removing a 447-bp *Eco*RV fragment. This maintains the open reading frame but removes 149 amino acids near the carboxy-terminal portion of the protein (Fig. 1). The proteins synthesized by the deleted clone were analyzed in an *E. coli* in vitro transcription-translation system (Fig. 4). The wild-type clone directed the synthesis of two proteins that presumably correspond to the precursor and processed forms of the pesticin receptor (Fig. 4, lane 1). Extracts containing the deleted version of the *psn* gene synthesized two smaller proteins, ~59 and 52 kDa in size (Fig. 4, lane 2). The difference in size from the wild-type proteins is consistent with a 149-amino-acid deletion. The mutated gene was introduced into the chromosome of *Y. pestis* KIM6+ and KIM10+ by allelic exchange, resulting in strains KIM6-2045.1 and KIM10-2045.5, respectively, in which the mutated gene has replaced the normal sequences. KIM10+, a strain that is missing the plasmid (pPCP1) encoding pesticin and its immunity protein, is sensitive to pesticin (48). However, KIM10-2045.5 was pesticin resistant, suggesting that the *psn* gene does encode the pesticin receptor.

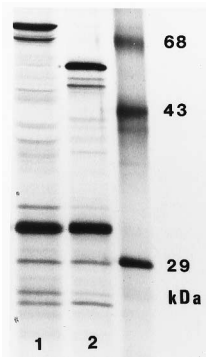

FIG. 4. Proteins synthesized in an in vitro transcription-translation assay containing plasmids bearing either the wild-type (pPSN13) or deleted (pPSN14) *psn* gene. ^{35}S-labeled proteins made in an *E. coli* S30 extract containing pPSN13 (lane 1) or pPSN14 (lane 2) were separated on denaturing 12% polyacrylamide gels and visualized by autoradiography. The molecular masses of ^{14}C-labeled standard proteins are indicated on the right.

Previous studies found that pesticin sensitivity correlates with the expression of a group of iron-regulated outer membrane proteins, IrpB to IrpE, that are 65 to 69 kDa in size and range from 5.37 to 5.98 in pI (58). Of these, only IrpB to IrpD are detected with ^{125}I by using surface-labeling techniques (46). To determine if the *psn* mutation alters the IrpB to IrpD proteins, we analyzed the proteins exposed on the surface of cells grown under iron-deficient conditions. The KIM6+ cells contained a characteristic three-lobed spot that presumably corresponds to the IrpB to IrpD proteins (Fig. 5A). These proteins were absent from KIM6+ cells grown in the presence of 10 μM $FeCl_3$ as well as from the Pgm$^-$ strain KIM6 grown in the presence and absence of iron (data not shown and reference 58). The IrpB to IrpD proteins were also missing from surface-labeled extracts of KIM6-2045.1 cells. Instead, we detected a new series of three spots that migrated faster and were slightly more acidic (Fig. 5B). This finding suggests that the pesticin receptor does correspond to the IrpB to IrpD proteins. For *Y. pestis*, we have retained the nomenclature of Sikkema and Brubaker (58) and used IrpC (the major protein) to designate the pesticin receptor. Presumably the single gene product migrates as three spots on isoelectric focusing gels as a result of translational errors and/or posttranslational modifications.

Total ^{35}S-labeled proteins synthesized by cells grown under iron-sufficient and iron-deficient conditions were analyzed by SDS-PAGE to determine the effect, if any, of the mutation on the expression of other iron-regulated proteins. As seen in Fig. 6, lanes 3 and 4, the iron-regulated synthesis of the two high-molecular-weight proteins, presumably HMWP1 and HMWP2 (8), as well as that of an ~56-kDa protein were unaffected by the *psn* mutation. However, synthesis of the 68-kDa iron-regulated pesticin receptor was abolished. All four of these iron-regulated proteins were present in KIM6+ as well as KIM6-2045, the cointegrant strain containing both a wild-type and mutant copy of the *psn* gene (Fig. 6, lanes 1, 2, 5, and 6). A new protein corresponding to the truncated version of the pesticin receptor/IrpC protein was not readily apparent on these gels. Perhaps the mutated protein is not as stable as the wild-type protein or it is masked by another protein with the same molecular weight.

Sikkema and Brubaker (58) showed that pesticin sensitivity in *Y. pestis* correlates not only with expression of IrpB to IrpE but also with the ability to grow in an iron-chelated medium at 37°C. In agreement with their results, we found that *Y. pestis* KIM6+, but not the *pgm* locus deletion mutant (KIM6) nor the *psn* mutant (KIM6-2045.1), was able to grow at 37°C on PMH-S plates. All three strains readily grew on this medium at 26°C. This finding suggests that the pesticin receptor, IrpC, is involved in accumulation of iron from a deficient or chelated environment at 37°C.

Construction and analysis of a *Y. pestis irp2* mutant. Other investigators have suggested that *irp2* may play a role in iron metabolism in *Y. enterocolitica* and *Y. pseudotuberculosis* (9, 27). To determine if *irp2* is involved in iron utilization in *Y. pestis*, we constructed an *irp2* mutation by inserting a kanamycin resistance cassette within the gene encoding the 190-kDa

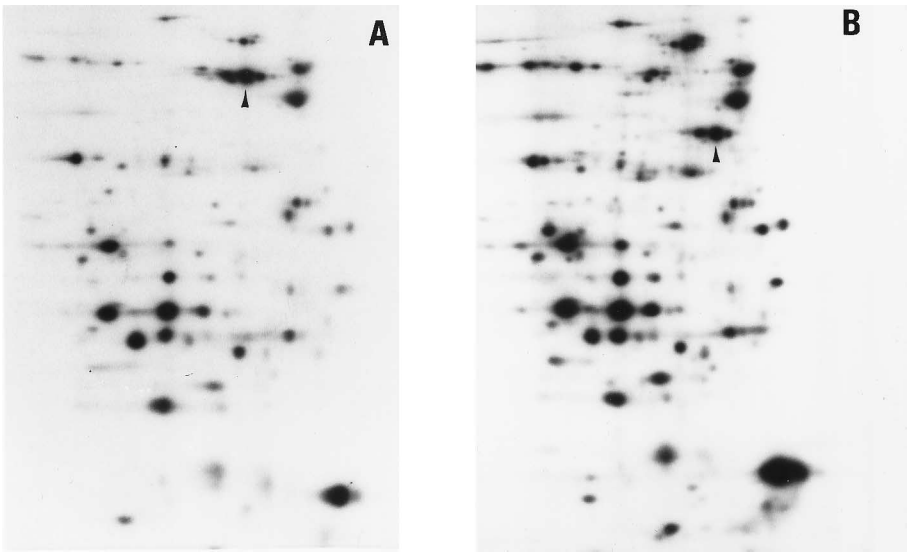

FIG. 5. Two-dimensional gel electropherograms of ^{125}I-labeled surface proteins from KIM6+ and the Δ*psn* mutant KIM6-2045.1. Surface-exposed proteins from KIM6+ (A) and KIM6-2045.1 (B) cells grown under iron-deficient conditions were labeled with ^{125}I and separated by isoelectric focusing followed by SDS-PAGE. Arrowheads point out the wild-type and modified IrpB to IrpD proteins.

protein, HMWP2. This construct was then used to replace the normal gene by allelic exchange, generating the *irp2* mutant strain KIM6-2046.1. In *Y. pseudotuberculosis*, inactivation of the *irp2* gene resulted in the loss of expression of both HMWP1 and HMWP2, suggesting that the genes for these proteins are part of the same operon (9). We obtained similar results with the *Y. pestis irp2* mutant. KIM6-2046.1 cells grown in the absence or presence of iron no longer synthesized the 240- and 190-kDa HMWP1 and HMWP2 proteins (Fig. 6, lanes 9 and 10). In addition, the ~56-kDa iron-regulated protein was missing and the level of IrpC was greatly reduced. The cointegrant strain KIM6-2046 synthesized the 190-kDa protein under iron-deficient conditions but not the 240- or 56-kDa protein (Fig. 6, lanes 7 and 8). These results suggest that in *Y. pestis*, like *Y. pseudotuberculosis*, the structural gene for the HMWP1 240-kDa protein (*irp1*) is part of an operon which includes *irp2* and possibly sequences encoding the 56-kDa protein.

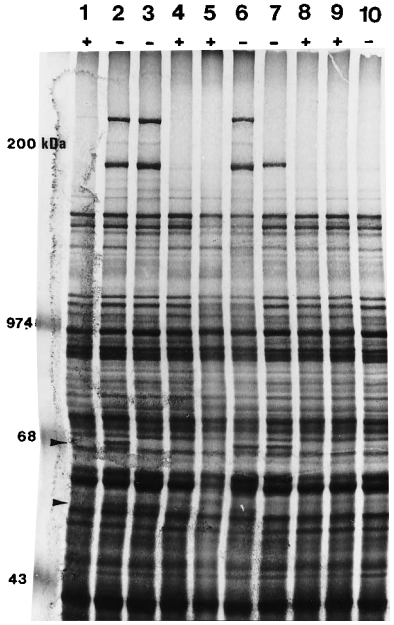

FIG. 6. Total proteins from *Y. pestis* strains grown under iron-sufficient or iron-deficient conditions. Cultures of *Y. pestis* KIM6-2045 (lanes 1 and 2), KIM6-2045.1 (lanes 3 and 4), KIM6+ (lanes 5 and 6), KIM6-2046 (lanes 7 and 8), and KIM6-2046.1 (lanes 9 and 10) were grown in the presence (+) or absence (−) of 10 μM $FeCl_3$ and incubated with ^{35}S-labeled amino acids. Total cellular proteins were separated on 7% polyacrylamide gels containing SDS and visualized by autoradiography. The masses of the standard proteins are indicated on the left. The arrowheads point to the 68- and 56-kDa iron-regulated proteins.

Although the *psn* gene possesses a functional promoter, the *irp2*::*kan* mutant consistently exhibited loss of IrpC (*psn*) expression (Fig. 6, lane 10; Fig. 7, lane 5). In contrast, the increased level of IrpC observed in the *irp2* cointegrant strain KIM6-2046 was highly variable (compare Fig. 6, lane 8, with Fig. 7, lane 6). As expected, complementation of KIM6-2046.1 with two different *irp2*-expressing recombinant plasmids restored iron-regulated expression of HMWP2 but not HMWP1 or the 56-kDa protein. However, neither of these in *trans*-complemented strains showed increased levels of IrpC expression (Fig. 7, lanes 1 to 4). The reasons for these variable results are currently unresolved.

The receptors for pseudobactin, pyoverdine, pyochelin, and enterobactin in *Pseudomonas* species and for ferric citrate in *E. coli* are induced by their cognate compounds (22, 34, 40, 50). Exposure of KIM6-2046.1 iron-deficient cells to spent supernatants from KIM6+ and KIM6-2045.1 (Fig. 7, lanes 10 and 8) stimulated expression of IrpC, while spent supernatants from KIM6 and KIM6-2046 did not influence IrpC expression. The effects of these supernatants on IrpC synthesis correlated with their abilities to stimulate growth of KIM6-2046.1 cells (see below).

Growth responses of *psn* and *irp2* mutants. Like the Δ*psn* mutant, the *irp2*::*kan* strain was unable to grow at 37°C on PMH-S plates but did grow on this medium at 26°C. At 37°C, KIM6+ was able to cross-feed the *irp2* mutant but not the Δ*psn* mutant (data not shown). To determine what products were required to restore growth to the *irp2* mutant, we performed a feeding assay. KIM6-2046.1 (*irp2*::*kan*) cells were used to overlay a PMH-S plate. Filtered supernatants from various cultures

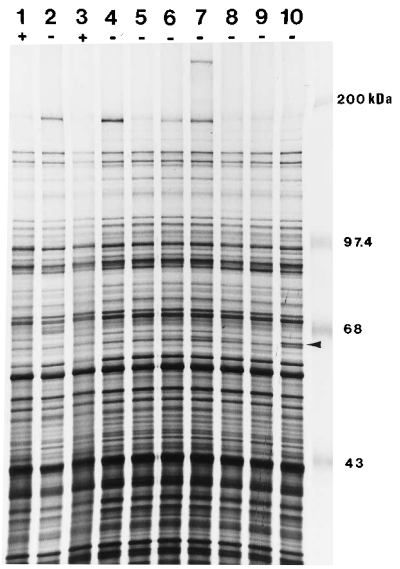

FIG. 7. Total proteins from *Y. pestis* strains grown under iron-sufficient or iron-deficient conditions. Cultures of *Y. pestis* KIM6-2046.1(pSDR498.8) (lanes 1 and 2) and KIM6-2046.1(pIRP2) (lanes 3 and 4) were grown in the presence (+) or absence (−) of 10 μM $FeCl_3$. Cultures of *Y. pestis* KIM6-2046 (lane 6), KIM6-2046.1 (lanes 5 and 8 to 10), and KIM6+ (lane 7) were cultivated without iron. KIM6-2046.1 cells were also exposed for 1.5 h to medium containing 50% spent supernatant from iron-deficient cultures of KIM6-2045.1 (lane 8), KIM6 (lane 9), or KIM6+ (lane 10). All cultures were incubated with ^{35}S-labeled amino acids. Total cellular proteins were separated on 7 to 12% linear gradient polyacrylamide gels containing SDS and visualized by autoradiography. The masses of the standard proteins are indicated on the right. The arrowhead identifies the 68-kDa IrpC protein.

grown under iron-deficient conditions were added to wells in the agar, and the plates were incubated at 37°C. Zones of growth were observed around wells containing supernatants from KIM10+, KIM6-2045.1 (*irp1*$^+$ *irp2*$^+$ Δ*psn*), KIM6-2030+ (*pgm*$^+$ *fur*::*kan-9*), KIM10(pSDR498) (Δ*pgm irp1*$^+$ *irp2*$^+$ *psn*$^+$), KIM6-2045 (*irp1*$^+$ *irp2*$^+$ *psn*::pPSN15), *Y. pseudotuberculosis* PB1/0, and *Y. enterocolitica* WA-LOX (data not shown). Culture filtrates from KIM10, KIM6-2046 (*irp2*::pCIRP498.8 *psn*$^+$), and KIM6-2046.1 (*irp2*::*kan psn*$^+$) were unable to restore growth to the *irp2* mutant strain. Furthermore, none of the supernatants were able to feed the Δ*psn* mutant (data not shown).

DISCUSSION

We have sequenced a region from the *pgm* locus of *Y. pestis* KIM6+ that renders certain *E. coli* and *Y. pestis* strains sensitive to the action of the bacteriocin pesticin. The *Y. pestis psn* gene is 98% identical to *fyuA* from *Y. enterocolitica*, which is believed to encode the receptor for pesticin as well as the siderophore yersiniabactin (52). The predicted amino acid sequences of the pesticin receptors from *Y. pestis* and *Y. enterocolitica* have regions that are homologous to other siderophore receptors, the most conserved domains being those that are probably involved in interactions with TonB (52). Pesticin sensitivity in *E. coli* (18) and *Y. enterocolitica*, as well as the uptake of yersiniabactin by *Y. enterocolitica*, is TonB dependent (30).

The promoter element of the *psn* gene contained an FBS which overlapped a putative −10 region and was 74% identical to the *E. coli* Fur-binding consensus sequence proposed by Pressler et al. (51). *Y. pestis* and *Y. enterocolitica* strains with mutations in *fur* are hypersensitive to pesticin, suggesting that Fur plays a role in regulating the expression of the pesticin receptor (30, 60, 62). A nearly perfect 14-bp inverted repeat was found upstream of the putative FBS in both *psn* and *fyuA* (52). A similar sequence is present upstream of the *irp2* gene in *Y. enterocolitica* (27) and probably *Y. pestis*. The precise function of this structure is unknown.

We constructed *Y. pestis* KIM6-2045.1 and KIM10-2045.5 containing an in-frame deletion in the *psn* gene. The mutant gene directed the synthesis of a truncated protein that is localized to the cell surface. Despite this, KIM10-2045.5 is not sensitive to pesticin. The Δ*psn* mutant did not express the IrpB to IrpD proteins but instead synthesized a new set of iron-regulated outer membrane proteins that migrated faster and were slightly more acidic. These results support the contention of Sikkema and Brubaker that the pesticin receptor corresponds to one or more of the IrpB to IrpE proteins (58). On one-dimensional SDS-polyacrylamide gels, the *psn* gene product migrates as a protein of ~68 kDa, which agrees well with the molecular mass predicted by sequence analysis. Presumably, the one gene product migrates as three spots on two-dimensional gels as a result of translational errors or posttranslational modifications. In keeping with the nomenclature of Sikkema and Brubaker (58), we have used IrpC to designate the pesticin receptor. We are unable to detect IrpE by ^{125}I surface-labeling techniques and do not know if it is encoded by a separate gene or is another modified form of IrpC.

We have also constructed *Y. pestis* strains containing mutations in *irp2*, the gene encoding the 190-kDa iron-regulated protein, HMWP2 (10, 19). The merodiploid strain KIM6-2046 still synthesized HMWP2 and variable levels of IrpC but failed to express two other iron-regulated proteins, 240 and ~56 kDa in size. Mutations in the *irp2* gene of *Y. enterocolitica* also eliminated synthesis of the 240-kDa protein, designated HMWP1 (9). These authors did not mention the 56-kDa protein. While it is possible that the 56-kDa protein is not present in *Y. enterocolitica*, this protein was difficult to resolve from other polypeptides on higher-percentage acrylamide gels. These results are consistent with the hypothesis that *irp2* is an upstream gene in an operon that also encodes HMWP1 (9) and the 56-kDa protein. Alternatively, mutations in *irp2* may have polar effects on a gene required for the transcriptional activation of the sequences encoding HMWP1 and the 56-kDa protein.

While the function of *irp2* is unknown, Guilvout et al. (27) speculate that HMWP2 in combination with HMWP1 may form a complex involved in the synthesis of an antibiotic, a new class of peptides, or the siderophore yersiniabactin. This speculation is based on an analysis of the *Y. enterocolitica irp2* sequence which revealed that HMWP2 is related to a group of adenylate-forming enzymes involved in the formation of nonribosomal peptide bonds (27). The small fragment of *Y. pestis irp2* that we have sequenced showed near identity to *Y. enterocolitica irp2*. Early studies indicated that *Y. pestis* cells produced a siderophore (69); however, subsequent analysis failed to detect siderophore activity in the yersiniae by either the Arnow or Csáky assay (11, 47). Using a chrome azurol S (CAS) assay (56), siderophore activity has been detected in *Y. pseudotuberculosis* (29) as well as *Y. enterocolitica* (11, 29). We have been unsuccessful in using the CAS assay to screen for siderophore production in *Y. pestis. Y. pestis* cells would not grow on CAS agar plates, and the liquid CAS assay yielded unreliable results (data not shown). Nonetheless, the cross-feeding studies presented here support the original notion (69) that *Y. pestis* produces a siderophore and suggest that HMWP1 and HMWP2 are involved in its synthesis. Although neither the Δ*psn* mutant (KIM6-2045.1) nor the *irp2* mutant (KIM6-

2046.1) was able to grow at 37°C on the iron-deficient PMH-S plates, supernatants from certain *Y. pestis* strains as well as *Y. pseudotuberculosis* PB1/0 and *Y. enterocolitica* WA-LOX restored growth of the *irp2* mutant. None of the supernatants were able to restore the growth of the Δ*psn* strain, suggesting that the receptor for the siderophore is encoded by the *psn* gene, in agreement with recent results for *Y. enterocolitica* which indicate that the pesticin receptor (FyuA) is also the receptor for yersiniabactin (52). Supernatants from the *irp2* merodiploid strain which produced HMWP2 and IrpC, but not HMWP1 and the 56-kDa iron-regulated protein, were unable to promote the growth of KIM6-2046.1. Thus, HMWP2 alone is not sufficient for the production of the siderophore. The 240- and 56-kDa iron-regulated proteins as well as other proteins, not yet identified, may also be required.

In addition to its likely involvement in the production of a siderophore, our results suggest that *irp2* may also play a role, directly or indirectly, in regulating the expression of the pesticin receptor (IrpC). When expression of HMWP2 is lost, as in strain KIM6-2046.1, the levels of IrpC are greatly reduced. In addition, we have previously noted that *Y. pestis* strains which have deleted the entire *pgm* locus, including the sequences encoding HMWP2, express very low levels of the IrpB to IrpD proteins when transformed with cloned DNA containing the *psn* gene (19). However, variable levels of IrpC are synthesized in the *irp2* merodiploid strain. HMWP2 has a helix-turn-helix motif, present in some DNA-binding proteins, and is homologous to AngR, a protein which transactivates certain genes involved in the synthesis of the *Vibrio anguillarum* siderophore anguibactin (27, 53). AngR does not, however, affect the synthesis of the anguibactin receptor FatA (66). Positive regulators required for maximal expression of siderophore receptors have been identified for several systems. The mechanisms involved appear to be as diverse as the siderophores themselves and include members of the two-component regulatory system (14), the LysR and AraC family of positive transcriptional activators (26, 31), as well as some potentially novel approaches (37, 68). Most of these receptors contain FBSs and are probably also controlled by Fur (2, 15, 25, 51). In some cases, the cognate siderophore is required to induce the expression of the receptor (22, 34, 40, 50).

To test the possibility that a *Y. pestis* siderophore induces synthesis of its receptor, we analyzed expression of IrpC in KIM6-2046.1 after exposure to spent supernatants from iron-deficient cultures of other *Y. pestis* strains. Supernatants which possessed growth-stimulatory activity for KIM6-2046.1, presumably due to the siderophore, also induced IrpC synthesis, while those lacking growth-stimulatory activity failed to increase IrpC expression. One anomaly which remains to be resolved is the variable expression of IrpC in the *irp2* merodiploid strain KIM6-2046. This strain, which expresses HMWP2 but not HMWP1 or the 56-kDa protein, cannot grow on PMH-S at 37°C presumably because it does not make the siderophore. It is possible that the siderophore precursor synthesized by this strain retains some regulatory activity but cannot deliver iron to the cell. The variable expression of IrpC in KIM6-2046 could be due to the residual regulatory activity of this precursor combined with differences in levels of expression of the precursor siderophore from experiment to experiment.

While it is tempting to speculate that the inverted repeats which lie upstream of *irp2* and the *psn* gene play a role in regulating their expression, further experimentation will be required to identify both the regulators and the regulatory sequences. However, our results suggest that *Y. pestis* does synthesize a siderophore and that the presence of this siderophore induces expression of its receptor, IrpC.

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