

Hierarchy of Iron Uptake Systems: Yfu and Yiu Are Functional in *Yersinia pestis*[∇]

Olga Kirillina, Alexander G. Bobrov, Jacqueline D. Fetherston, and Robert D. Perry*

Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, Kentucky

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In addition to the yersiniabactin (Ybt) siderophore-dependent system, two inorganic iron ABC transport systems of *Yersinia pestis*, Yfe and Yfu, have been characterized. Here we show that the Yfu system functions in *Y. pestis*: a Ybt⁻ Yfe⁻ Yfu⁻ mutant exhibited a greater growth defect under iron-deficient conditions than its Ybt⁻ Yfe⁻ parental strain. We also demonstrate that another putative *Y. pestis* iron uptake system, Yiu, which potentially encodes an outer membrane receptor, YiuR, and an ABC iron transport cassette, YiuABC, is functional. The cloned *yiABC* operon restored growth of an enterobactin-deficient mutant *Escherichia coli* strain, 1017, under iron-chelated conditions. Iron uptake by the Yiu system in *Y. pestis* was demonstrated only when the Ybt, Yfe, and Yfu systems were mutated. Using a *yiA::lacZ* fusion, we show that the *yiABC* promoter is repressed by iron through Fur. A mouse model of bubonic plague failed to show a significant role for the Yiu system in the disease process. These results demonstrate that two additional iron transporters are functional in *Y. pestis* and indicate that there is a hierarchy of iron transporters, with Ybt being most effective and Yiu being the least effective of those systems which have been characterized.

Iron is a vital element for the vast majority of microorganisms due to its role in redox reactions. However, inorganic iron is readily available neither in aerobic environments nor in biological systems. In potential hosts, iron is located intracellularly, chelated by high-affinity iron binding proteins such as transferrin and lactoferrin, or bound in heme and hemoproteins. Thus, iron sequestration is an innate host defense against invading pathogens. Consequently pathogenic, as well as saprophytic, bacteria have evolved a large variety of iron acquisition strategies for their survival and growth. Production and use of siderophores (low-molecular-weight high-affinity iron ligands), outer membrane (OM) receptors for binding host iron-chelating proteins, and ferrous iron transporters, as well as transport systems for heme and hemoproteins, are common iron acquisition methods. After transporting various iron complexes through the bacterial OM, most systems use ABC transporters to mediate transport through the periplasm and inner membrane into the cytoplasm. ABC uptake systems generally possess a ligand-binding protein (in the periplasm in gram-negative bacteria), an inner membrane permease, and an ATP hydrolase (9, 13, 49).

Like many bacteria, including *Escherichia coli*, *Salmonella*, *Shigella*, and *Vibrio* species (17, 31), *Yersinia pestis*, the etiologic agent of bubonic and pneumonic plague, encodes multiple iron and heme uptake systems (32, 35). The yersiniabactin (Ybt) system produces the Ybt siderophore via a nonribosomal peptide/polyketide synthesis system, secretes the siderophore, and utilizes iron from the Fe-Ybt complex by transport through the OM, TonB-dependent receptor Psn, and the YbtPQ ABC transporter. The Ybt system is encoded on a pathogenicity

island within the 102-kb *pgm* locus, which undergoes spontaneous deletion. Since a functional Ybt system is absolutely required for plague infection by subcutaneous or peritoneal routes, it is likely a major mechanism of iron acquisition by *Y. pestis* during the early stages of bubonic plague. However, the Ybt system does not appear to play any important role during the later stages of plague—Ybt⁻ mutants remain fully virulent by an intravenous route of infection (4, 8, 20, 28, 32, 35).

The TonB-independent Yfe ABC transport system for iron and manganese uptake is necessary for full virulence in a bubonic plague model. A Yfe⁻ mutant causes an ~5- to 75-fold decrease in the 50% lethal dose (LD₅₀) by a subcutaneous route of infection, depending upon the strain background, compared to its Yfe⁺ parental strain. A Ybt⁻ Yfe⁻ double mutant was completely avirulent by an intravenous route of infection. This suggests that the Ybt and Yfe systems are the primary iron acquisition systems for *Y. pestis* during the course of bubonic plague (5, 6, 25, 32, 35).

Gong et al. (25) showed that the *Y. pestis* Yfu ABC transporter restored the ability of an *E. coli* enterobactin-deficient mutant to grow in an iron-chelated medium and is iron and Fur regulated in *Y. pestis*. While this indicates that Yfu functions to transport iron similar to its orthologue in *Yersinia enterocolitica* (40), *Y. pestis* Yfu⁻ mutants did not display a growth-defective phenotype in vitro and were fully virulent in a mouse model of bubonic plague (25).

In addition to these proven iron transporters, the *Y. pestis* KIM and CO92 genomes contain five other putative iron/siderophore ABC transporters (two associated with genes encoding potential siderophore biosynthetic enzymes) and a putative ferrous iron transporter (FeoABC) (32, 35). In this study, we demonstrate that the previously identified Yfu system, as well as another ABC transporter, Yiu, functions in *Y. pestis*. In vitro growth-defective phenotypes of the Δ *yiABC*R mutant are apparent under iron-chelated conditions in a Ybt⁻ Yfe⁻ Yfu⁻

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, KY 40656-0298. Phone: (859) 323-6341. Fax: (859) 257-8994. E-mail: rperry@uky.edu.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	Cloning strain	2
DH5 α - λ pir	Strain for propagating plasmids with R6K origins; derived from DH5 α	S. C. Straley
1017	ent::Tn5 Km ^r ; derived from HB101	14
<i>Y. pestis</i>		
KIM5-2082.3(pCDAp1)+	Pgm ⁺ (Ybt ⁺) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁻ (Δ yfuA2082) Yiu ⁺ Lcr ⁺ (<i>yadA::bla</i> ; Ap ^r); pMT1, pPCP1, pCD1Ap	25
KIM5-2123.2(pCDAp1)+	Pgm ⁺ (Ybt ⁺) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁻ (Δ yfuA2082) Yiu ⁻ (Δ yiuABCR2123) Lcr ⁺ (<i>yadA::bla</i> ; Ap ^r); pMT1, pPCP1, pCD1Ap	This study
KIM6+	Pgm ⁺ (Ybt ⁺) Yfe ⁺ Yfu ⁺ Yiu ⁺ Lcr ⁻ ; pMT1, pPCP1	23
KIM6	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁺ Yfu ⁺ Yiu ⁺ Lcr ⁻ ; pMT1, pPCP1	23
KIM6-2030	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁺ Yfu ⁺ Yiu ⁺ Fur ⁻ (<i>fur::kan-9</i>) Lcr ⁻ ; pMT1, pPCP1	44
KIM6-2031.1	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁺ Yiu ⁺ Lcr ⁻ ; pMT1, pPCP1	5
KIM6-2082.1	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁻ (Δ yfuA2082) Yiu ⁺ Lcr ⁻ ; pMT1, pPCP1	25
KIM6-2123+	Pgm ⁺ (Ybt ⁺) Yfe ⁺ Yfu ⁺ Yiu ⁻ (Δ yiuABCR2123) Lcr ⁻ ; pMT1, pPCP1	This study
KIM6-2123	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁺ Yfu ⁺ Yiu ⁻ (Δ yiuABCR2123) Lcr ⁻ ; pMT1, pPCP1	This study
KIM6-2123.1	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁺ Yiu ⁻ (Δ yiuABCR2123) Lcr ⁻ ; pMT1, pPCP1	This study
KIM6-2123.2	Pgm ⁻ (Δ pgm, Ybt ⁻) Yfe ⁻ (Δ yfeAB2031.1) Yfu ⁻ (Δ yfuA2082) Yiu ⁻ (Δ yiuABCR2123) Lcr ⁻ ; pMT1, pPCP1	This study
KIM10+	Pgm ⁺ (Ybt ⁺) Yfe ⁺ Yfu ⁺ Yiu ⁺ Lcr ⁻ ; pMT1; KIM6+ cured of pPCP1	22
Plasmids		
pET24A+	5.3-kb expression vector; Km ^r	Novagen
pEU730	15.2-kb single-copy-no. reporter vector, promoterless <i>lacZ</i> ; Spc ^r	24
pKNG101	6.8-kb suicide vector, SacB+ R6K origin; Sm ^r	27
pLC8.2	8.2-kb single-copy cloning vector; Ap ^r	26
pUC19	2.7-kb high-copy-no. cloning vector; Ap ^r	2
pWSK29	5.4-kb low-copy-no. cloning vector; Ap ^r	48
pYIU1	9.65 kb, Yiu ⁺ Ap ^r ; 6.95-kb SacI-SphI fragment from <i>Y. pestis</i> KIM10+ genomic DNA ligated into pUC19	This study
pYIU2	11.7 kb, Yiu ⁺ Ap ^r ; 6.316-kb SacI-PstI fragment from pYIU1 ligated into pWSK29	This study
pYIU3	6.5 kb, <i>yiuABC</i> ⁺ Ap ^r ; 3.15-kb PvuII fragment deleted from pYIU2	This study
pYIU4	7.8 kb, <i>yiuA</i> ⁺ Ap ^r ; 2.395-kb EcoRI fragment from pYIU2 ligated into pWSK29	This study
pYIU5	10.4 kb, <i>yiuBC</i> ⁺ Ap ^r ; 4.98-kb BamHI fragment from pYIU2 ligated into pWSK29	This study
pYIU6	8.0 kb, <i>yiuR</i> ⁺ Ap ^r ; 2.6-kb SspI-PstI fragment from pYIU2 ligated into pWSK29	This study
pYIU7	9.6 kb, <i>yiuA::lacZ</i> Ap ^r ; 5.4-kb BamHI-EcoRV fragment deleted from pYIU2 and replaced with 3.342-kb BamHI-NruI fragment with promoterless <i>lacZ</i> gene from pEU730	This study
pYIU8	12.4 kb, <i>yiuA::lacZ</i> Ap ^r ; 4.18-kb EcoRI-XhoI fragment from pYIU7 ligated into pLC8.2	This study
pYIU9	4.7 kb, Δ yiuABCR2123 Ap ^r ; 4.98-kb BamHI fragment deleted from pYIU1	This study
pYIU10	6.9 kb, Δ yiuABCR2123 Km ^r ; 1.97-kb SacI-SphI fragment from pYIU9 ligated into pET24A+	This study
pYIU11	9.1 kb, Δ yiuABCR2123 Sm ^r ; 2.6-kb ApaI-SalI fragment from pYIU10 ligated into pKNG101	This study

^a *Y. pestis* strains lacking pCD1Ap are avirulent due to lack of this low-calcium-response plasmid. Strains with a plus sign possess an intact 102-kb *pgm* locus containing the genes for biofilm formation (*hms*) and the Ybt iron transport system. All other *Y. pestis* strains have either a *pgm* deletion or a mutation within the *pgm* locus. Abbreviations Ap^r, Km^r, Sm^r, and Spc^r indicate resistance to ampicillin, kanamycin, streptomycin, and spectinomycin, respectively.

background. In vivo, the Δ yiuABCR mutation failed to show a significant loss of virulence in a bubonic plague model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. The relevant characteristics of all plasmids and bacterial strains used in this study are given in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani broth at 37°C. Iron-deficient growth of *E. coli* strains was assessed in nutrient broth (Difco Laboratories) supplemented with 50 μ M 2,2'-dipyridyl (DIP). After acclimation to these iron-chelated conditions, the growth of *E. coli* strains was monitored at the optical density at 620 nm (OD₆₂₀). *Yersinia pestis* strains were cultured in heart infusion broth (HIB)

and on tryptose blood agar base (TBA) or Congo red plates at 30 and 37°C. Growth in liquid medium was monitored with a Genesys 5 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY). Where necessary, ampicillin (Ap), kanamycin (Km), streptomycin (Sm), and spectinomycin (Spc) were used at final concentrations of 100, 50, 50, and 100 μ g/ml, respectively. For some experiments, media were supplemented with 40 μ g 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)/ml.

Y. pestis strains were grown in the chemically defined medium PMH or PMH2, deferrated prior to use with Chelex 100 resin (Bio-Rad Laboratories) (25, 45). For some studies, PMH2 medium was supplemented with either DIP or ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDA) to chelate iron in the medium. After overnight incubation at 30 or 37°C on a TBA slant, cells were

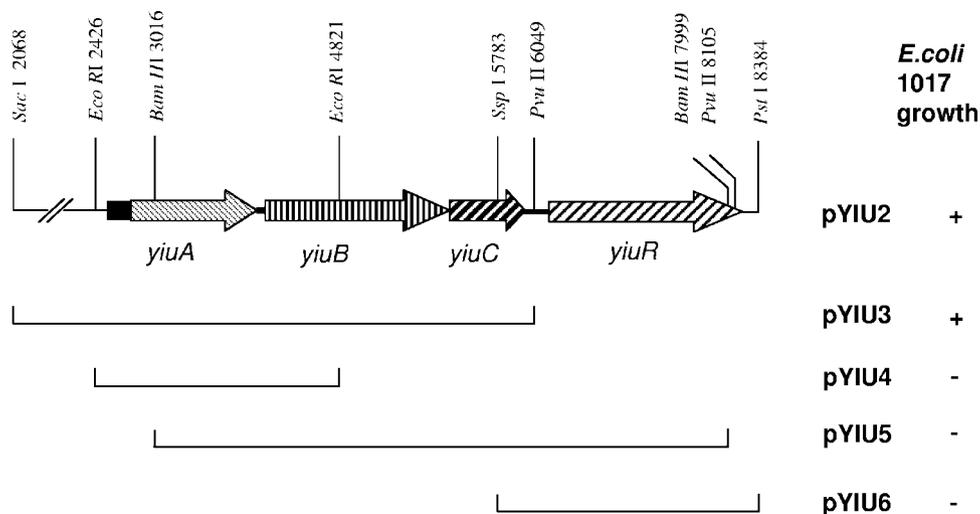


FIG. 1. Genetic organization of the *yiu* locus of *Yersinia pestis* and maps of recombinant pYIU plasmids. Arrows indicate the direction of transcription of *yiu* genes. A putative promoter and overlapping Fur-binding site are shown as a black box. The $\Delta yiuABCR2123$ mutation lacks the ~5-kb BamHI fragment. Symbols: +, plasmids which promote iron-chelated growth of *E. coli* 1017; -, no iron-chelated growth stimulation of *E. coli* 1017.

suspended in PMH2 to an OD_{620} of 0.1 and grown with aeration at 37°C for 8 h prior to transfer to fresh PMH2 (OD_{620} of 0.1) and grown under the same conditions overnight. Cells from the overnight cultures were transferred to fresh PMH2 (OD_{620} of 0.1, 0.05, or 0.005), and growth was monitored with a Genesys spectrophotometer (Spectronic Instruments, Inc.) at regular intervals.

As previously described, gradient plates with iron-chelated PMH2 were used to compare the growth of *Y. pestis* mutants (5). Gradients of 0 to 25 μ M DIP or 0 to 1 μ M EDDA were prepared to streak 1.5×10^5 cells acclimated to iron-deficient growth conditions as described above. PMH2 was solidified with 1% agarose. Ampicillin at 25 μ g/ml was added to PMH2, and a gradient of 0 to 12.5 μ M DIP was prepared for complementation analysis of KIM6-2123.2 ($\Delta yiuABCR2123.2$). Growth into the increasing chelator gradient was monitored daily for a period of 72 h at 37°C.

Recombinant DNA techniques. Plasmid DNA was isolated by alkaline lysis (7) and transformed into *E. coli* strains by a standard $CaCl_2$ method (41). Transformation of plasmid DNA into *Y. pestis* strains was accomplished by electroporation as previously described (21). Bacterial genomic DNA was isolated by the *N*-cetyl-*N,N,N*-trimethyl-ammonium bromide method (2). Restriction endonuclease digestions and experiments requiring the use of DNA-modifying enzymes were performed according to the recommendations of the commercial supplier (New England Biolabs and MBI Fermentas).

Sequence analysis. Nucleotide sequences were analyzed for promoters using the web-based program BPROM (www.softberry.com). Predictions of potential Fur binding sites used the original 19-bp inverted repeat as well as proposed hexamer repeats and overlapping heptamer inverted repeats (3, 15). Amino acid homology searches were conducted using the BLAST algorithms available at the National Center for Biotechnology Information (1). Protein alignments and similarities were analyzed using CLUSTALW multiple sequence alignment (11). SignalP 3.0 was used to predict signal sequence cleavage sites (19).

Construction of recombinant *yiuABCR* plasmids and *Y. pestis* mutants. To clone the *Y. pestis yiu* genes (Y2872 to -2875), genomic DNA from KIM10+ was digested with SacI and SphI endonucleases. Fragments of ~6 to 8 kb were isolated from low-melting agarose, cloned into pUC19, and transformed in *E. coli* DH5 α . Clones carrying *yiu* genes were detected by PCR using primers YIU-1 (5'-GTATTGGCGCATTCTATCCGTG-3') and YIU-2 (5'-ATATCAC CACAAATACGACTGGGC-3'). Reactions were performed in a GeneAmp PCR System 2400 (Perkin-Elmer) and run for 5 min at 94°C and then for 30 cycles at 94°C for 30 s, 30 s at 55°C, and 30 s at 72°C, followed by a single cycle at 72°C for 7 min. One clone containing the desired insert was designated pYIU1. A DNA fragment containing *yiuABCR* from pYIU1 was cloned into pWSK29, generating pYIU2. Fragments from pYIU2 were used to construct recombinant plasmids containing intact *yiuABC* (pYIU3), *yiuA* (pYIU4), *yiuBC* (pYIU5), and *yiuR* (pYIU6) (Table 1).

A deletion of the *yiuA*, *-B*, *-C*, and *-R* genes was made by eliminating a 4,980-bp BamHI fragment (Fig. 1) from pYIU2 to generate pYIU9. A fragment

containing the $\Delta yiuABCR2123$ mutation was ligated into pET24A+, generating pYIU10, to obtain appropriate restriction enzyme sites for cloning the $\Delta yiuABCR2123$ fragment into the suicide vector pKNG101. The resulting plasmid, pYIU11, propagated in *E. coli* DH5 α *lpir*, was electroporated into *Y. pestis* KIM6+ (Pgm+ [Ybt+]), KIM6 (Δpgm [Ybt-]), KIM6-2031.1 ($\Delta pgm \Delta yfeAB2031.1$), and KIM6-2082.1 ($\Delta pgm \Delta yfeAB2031.1 \Delta yfuAB2082$). Cells from Sm^r colonies were grown overnight in HIB without Sm to identify sucrose-resistant isolates that had completed the allelic exchange as described previously (5). PCR using primers YIU-1 and YIU-3 (5'-TATCCACACGCTTATCCAAC AGGT-3') confirmed the $\Delta yiuABCR$ mutation, and one isolate from each strain was designated KIM6-2123+ (Pgm+ $\Delta yiuABCR2123$), KIM6-2123 ($\Delta pgm \Delta yiuABCR2123$), KIM6-2123.1 ($\Delta pgm \Delta yiuABCR2123 \Delta yfeAB2031.1$), or KIM6-2123.2 ($\Delta pgm \Delta yiuABCR2123 \Delta yfeAB2031.1 \Delta yfuAB2082$) (Table 1).

Construction of a *yiuA::lacZ* reporter plasmid and β -galactosidase assays. A 5.4-kb BamHI-EcoRV fragment deleted from pYIU2 was replaced with a 3.342-kp BamI-NruI fragment from pEU730. The resulting plasmid contains the promoterless *lacZ* gene fused to the *yiuA* promoter and was designated pYIU7. A 4.18-kb EcoRI-XhoI fragment from pYIU7 was cloned into the single-copy vector pLC8.2, generating pYIU8 (Table 1). This reporter plasmid was transformed into *Y. pestis* strains to analyze transcriptional regulation of the *yiuA* promoter. *Y. pestis* strains containing the reporter plasmid were acclimated to iron-deficient or iron-surplus conditions (~6 generations in PMH2 without added iron or with 10 μ M $FeCl_3$) and harvested during exponential growth at 37°C. β -Galactosidase activities from whole-cell lysates were measured spectrophotometrically with a Genesys5 spectrophotometer following cleavage of *o*-nitrophenyl- β -D-galactopyranoside (ONPG), and the results are expressed in Miller units (29a). The data presented are the averages and standard deviations derived from five independent experiments.

Virulence testing. To generate a *yfe yfu yiu* triple-mutant strain for virulence testing in mice, pCD1Ap was electroporated into KIM6-2123.2+ (Ybt+ Yfe- Yfu- Yiu-) in the CDC-approved University of Kentucky BSL3/ABSL3 facility. The presence of pCD1Ap was confirmed by gel electrophoresis of total plasmid DNA, and the resulting strain was designated KIM5-2123.2(pCD1Ap)+. Pgm+ and Lcr+ phenotypes were confirmed, respectively, on Congo red plates (46) and TBA plates supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$ (34). KIM5-2082.1(pCD1Ap)+ (Ybt+ Yfe- Yfu-) and KIM5-2123.2(pCD1Ap)+ (Ybt+ Yfe- Yfu- Yiu-) were grown at 26 to 30°C in HIB supplemented with Ap (50 μ g/ml); exponential-phase cells were diluted in mouse isotonic phosphate-buffered saline (149 mM NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 [pH 7.0]). Five- to seven-week-old female Swiss Webster mice were injected subcutaneously with 0.1 ml of 10-fold serial dilutions of the bacterial suspensions. Four mice were used for each bacterial dose. The number of cells injected was determined by plating serial dilutions on TBA-Ap plates. Mice were monitored daily for a period of 2 weeks.

RESULTS

Sequence analysis of *yi*u genes. We searched the *Y. pestis* CO92 and KIM10+ genomes for homologues to iron transport proteins from other bacteria and identified a four-gene locus, Y2872 to -Y2875 in KIM10+ and YPO1310 to YPO1313 in CO92 (16, 30), which we have designated *yi*u, for *Yersinia* iron uptake (Fig. 1). An NCBI conserved domain search revealed that YiuA (Y2875) has a periplasmic binding protein domain of the TroA_f family, whose members include periplasmic binding proteins for a variety of bacterial iron transport systems. SignalP 3.0 (19) predicts a signal sequence cleavage site that would generate a processed YiuA protein of 351 amino acids. The annotated methionine and valine starts in KIM10+ and CO92, respectively, would yield 38- or 30-amino-acid signal peptides. A third possible methionine start would result in a 23-amino-acid signal peptide. Given that the only strong potential ribosome binding site is associated with the valine initiation codon, the annotated start in CO92 may be correct. The second gene in the putative operon, *yi*uB (y2874), is predicted to be an inner membrane permease of the FecCD family with nine putative transmembrane domains. The third open reading frame (ORF), YiuC, is a putative ATP binding protein containing typical Walker boxes with a COG1120 conserved domain that is also present on ATP binding proteins for cobalamin and some siderophores. YiuA, YiuB, and YiuC have significant similarities (35 to 45% identity) to Irp6A, Irp6B, and Irp6C, a siderophore-dependent iron uptake system in *Corynebacterium diphtheriae* (37). The fourth *Y. pestis* gene, *yi*uR, encodes a putative OM TonB-dependent receptor sharing 45% identity with the *Vibrio cholerae* enterobactin receptor IrgA as well as 38% and 30% identities with the *E. coli* colicin I receptor and FepA, the receptor for ferrienterobactin. Removal of a signal sequence cleavage predicted by SignalP 3.0 (19) would generate a processed YiuR protein of 637 amino acids.

This four-gene locus is also present in the genomes of *Y. pestis* 91001 (43), *Y. pestis* Angola, *Y. pestis* Pestoides F, *Yersinia pseudotuberculosis* IP32953 (10), *Y. pseudotuberculosis* PB1, and *Photobacterium luminescens* subsp. *laumondii* TTO1 (18). All four ORFs have 100% predicted amino acid identity in all five *Y. pestis* genomes and 99 to 100% identity to the *Y. pseudotuberculosis* ORFs. They show 79 to 93% amino acid similarity to the *P. luminescens* ORFs. BLAST analysis of the genome sequence database of *Yersinia enterocolitica* 8081 at the Sanger Centre showed that *yi*uB, *yi*uC, and *yi*uR are present, but *yi*uA was not identified in the unfinished genome.

The *yi*u genes enhance the iron-deficient growth of *E. coli* 1017. *E. coli* 1017, an enterobactin-deficient (Ent⁻) Tn5 insertion mutant of HB101, grows poorly under iron-depleted, but not under iron-surplus, conditions and has been used successfully to identify a number of iron uptake systems from various bacteria (14, 39). To determine whether the entire *yi*u locus as well as individual *yi*u genes could function in iron uptake, we transferred various combinations of the *yi*u genes cloned in pWSK29 into *E. coli* 1017. The iron-chelated growth of *E. coli* 1017 carrying pYIU2 (*yi*uABC⁺), pYIU3 (*yi*uABC⁺), pYIU4 (*yi*uA⁺), pYIU5 (*yi*uBC⁺), or pYIU6 (*yi*uR⁺) was compared to that of 1017 carrying the vector plasmid pWSK29 (Fig. 1). The iron-chelated growth of *E. coli* 1017 was substantially pro-

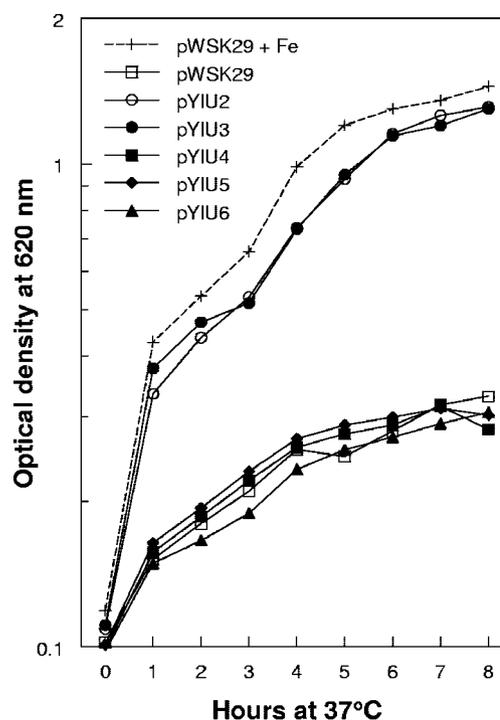


FIG. 2. Iron-chelated growth of *E. coli* 1017 transformed with plasmids carrying various *yi*u genes. Strains were grown in NB supplemented with 50 μ M DIP at 37°C. The vector plasmid for all constructs was pWSK29. + Fe indicates growth of *E. coli* (pWSK29) with 25 μ M FeCl₃. The following plasmids were used: pYIU2 (*yi*uABC⁺), pYIU3 (*yi*uABC⁺), pYIU4 (*yi*uA⁺), pYIU5 (*yi*uBC⁺), and pYIU6 (*yi*uR⁺). The growth curves shown represent one of two independent experiments that yielded similar results.

moted when the entire *yi*u locus (pYIU2) or just the *yi*uABC genes (pYIU3) were present; growth was not enhanced in 1017 strains carrying *yi*uA (pYIU4), *yi*uBC (pYIU5), or *yi*uR (pYIU6) (Fig. 2). These data suggest that the *yi*uABC genes encode an ABC iron transporter that can function in *E. coli* 1017 as a unit independently from the genetically linked gene for the OM receptor YiuR, at least under the iron-chelating conditions tested here. In *E. coli* K-12, the colicin I receptor has the highest similarity (55%) to YiuR, followed by FepA (45%). Given this low degree of similarity, it seems unlikely that these receptors function in place of YiuR in *E. coli*. Perhaps the YiuABC transporter, like the Fhu system, is able to transport Fe derived from other OM receptors, or perhaps the Yiu system uses a porin.

Regulation of the *yi*uA promoter by iron and Fur. Generally, transcription of genes encoding iron uptake systems is repressed by iron-surplus conditions. In most gram-negative bacteria, a Fur-Fe²⁺ dimer binds to a conserved DNA sequence, called a Fur box, which usually overlaps the -10 and/or -35 promoter motifs, blocking access of RNA polymerase to the promoter. We identified a potential Fur box (GATAAgtATtATCATTtGc) that overlaps the putative -35 promoter sequence of *yi*uA and matches the consensus in 14 of 19 bp (nonmatching residues in lowercase). To test whether this promoter is iron and Fur regulated, we constructed a *yi*uA::lacZ transcriptional fusion in a single-copy vector, pYIU8. Cells of

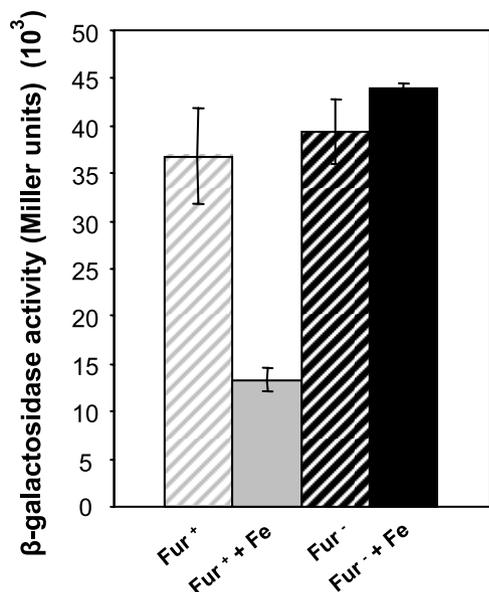


FIG. 3. Iron- and Fur-dependent regulation of the *yiuA* promoter. *Y. pestis* Fur⁺ or Fur⁻ strains (KIM6 and KIM6-2030) carrying pYIU8 (*yiuA::lacZ*) were grown in iron-depleted PMH medium at 37°C with and without 10 μ M added ferric chloride. The values are averages of replicate samples from five independent experiments. Error bars indicate standard deviations.

KIM6(pYIU8) and KIM6-2030(pYIU8) (*fur::kan*) were grown at 37°C in iron-deficient PMH medium in the presence or absence of added iron and assayed for β -galactosidase activity. The expression of *yiuA::lacZ* in KIM6 grown with surplus iron was repressed threefold compared to that of the iron-deficient culture. In the absence of Fur, the *yiuA::lacZ* promoter was no longer repressed by surplus iron in the medium (Fig. 3). Thus, the *yiuA* promoter requires a functional Fur protein for repression by iron.

Iron-deficient and iron-chelated growth of *yfu* and *yiu* mutants. To determine whether the *yiu* system functions in iron acquisition in *Y. pestis*, we constructed a $\Delta yiuABC$ mutation in KIM6⁺ and designated the resulting strain KIM6-2123+ (Ybt⁺ Yfe⁺ Yfu⁺ Yiu⁻) (Table 1). Under iron-deficient growth conditions in PMH2, there was no significant difference in the growth of the parent and mutant strains (data not shown). Similarly, the mutant and parental strains grew into the same concentration of DIP or EDDA on gradient plates where cells are exposed to progressively higher concentrations of the iron chelator (data not shown).

Previous studies with the Yfe ABC transporter showed that the Ybt siderophore-dependent iron transport system masked growth defects caused by *yfe* mutations (5). Consequently, we tested whether the Yfe and/or Yfu transporters might have a similar effect on the Yiu system by introducing the $\Delta yiuABC$ 2123 mutation into KIM6 (Ybt⁻), KIM6-2031.1 (Ybt⁻ Yfe⁻ Yfu⁺), and KIM6-2082.1 (Ybt⁻ Yfe⁻ Yfu⁻). Using a typical inoculation density for *Y. pestis* (OD₆₂₀ of 0.05 to 0.1), all strains yielded similar growth profiles under iron-deficient conditions (data not shown). However, growth initiated from an OD₆₂₀ of 0.005 showed a significant growth defect due to the $\Delta yiuA2082$ mutation in a Yfe⁻ background.

Under these conditions, the $\Delta yiuABC$ 2123 mutation did not cause a further loss of growth in a Yfe⁻ Yfu⁻ background (Fig. 4A). This experiment indicates that the Yfu system does function to acquire iron in *Y. pestis*.

Upon further iron restriction resulting from the presence of 45 μ M DIP under similar growth conditions, the Ybt⁻ Yfe⁻ Yfu⁻ Yiu⁻ quadruple mutant displayed a modest increase in generation time and decrease in final cell yield compared to its Ybt⁻ Yfe⁻ Yfu⁻ Yiu⁺ isogenic parent (Fig. 4B). Finally, addition of iron to the chelated growth medium restored growth of both the Ybt⁻ Yfe⁻ Yfu⁻ Yiu⁺ and Ybt⁻ Yfe⁻ Yfu⁻ Yiu⁻ mutants to levels comparable to those of their parental strains (data not shown).

Plates containing gradients of 0 to 25 μ M DIP or 0 to 1 μ M EDDA were used to reveal growth defects due to mutations in the Yfu or Yiu systems. At 37°C, cells of KIM6-2031.1 (Ybt⁻ Yfe⁻ Yfu⁺) grew to approximately twofold higher DIP or EDDA concentrations than did KIM6-2082.1 (Ybt⁻ Yfe⁻ Yfu⁻) cells (Fig. 5A and B). The $\Delta yiuABC$ 2123 mutation in a Ybt⁻ Yfe⁻ Yfu⁻ background caused a twofold loss of growth across the DIP gradient compared to KIM6-2082.1 (Ybt⁻ Yfe⁻ Yfu⁻) (Fig. 5A). Under these conditions, EDDA nearly eliminated the ability of KIM6-2123.2 (Ybt⁻ Yfe⁻ Yfu⁻ Yiu⁻) cells to grow (Fig. 5B). These results demonstrate that the Yiu system does function in vitro as an iron uptake system in *Y. pestis*.

The *yiuABC* mutation was complemented in *trans* by recombinant plasmid with all *yiu* genes (pYIU2). Expression of *yiuABC* genes from low-copy vector plasmid restored growth of *Y. pestis* KIM6-2123.2 ($\Delta yiuABC$ 2123.2) on 0 to 12.5 and 0 to 25 μ M DIP gradient plates to a level exceeding that of the parental strain. *Y. pestis* KIM6-2123.2 transformed with the vector plasmid pWSK29 did not increase the iron-chelated growth of the *yiuABC* mutant (data not shown).

The role of the Yiu iron transporter in the virulence of bubonic plague. Previously, we demonstrated that Ybt⁻ mutants are completely avirulent from a subcutaneous site of infection mimicking bubonic plague in a mouse model and that a Yfe⁻ mutant had a modest increase in the LD₅₀ in this model. However, a Yfe⁻ Yfu⁻ mutant displayed no further loss of virulence (4, 5, 20, 25). To test whether a *Y. pestis* strain lacking three iron/siderophore ABC transporters lost virulence, we transformed KIM6-2123.2 (Ybt⁺ Yfe⁻ [*yfeAB2031.1*] Yfu⁻ [*yfuA2082*] Yiu⁻ [$\Delta yiuABC$ 2123]) with pCD1Ap and determined the LD₅₀ of this strain in a mouse model of bubonic plague. The LD₅₀ of our new triple mutant did not differ significantly from the Ybt⁺ Yfe⁻ Yfu⁻ double mutant. This indicates that the Yiu system does not play a significant role in the virulence of bubonic plague that can be measured by this method.

DISCUSSION

Y. pestis, like a number of other gram-negative pathogens (e.g., *E. coli*, *Shigella*, *Neisseria*, *Pseudomonas*, and *Vibrio*), possesses multiple proven or putative iron transport systems (12, 17, 31, 32, 35, 36). The relatively well-characterized Ybt system produces a siderophore composed of salicylate, thiazoline, and thiazolidine rings with a high affinity for ferric iron (10⁻³⁶) and is essential for the virulence of plague from pe-

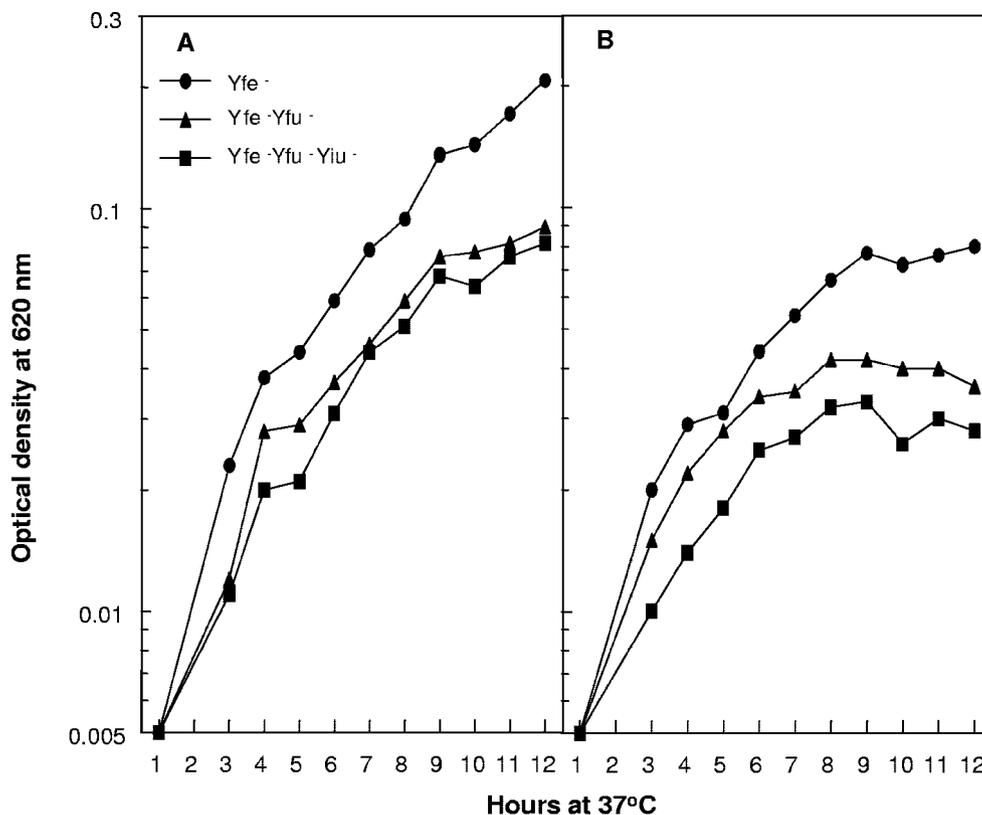


FIG. 4. Effect of Δyfu and Δyiu mutations on iron-deficient or iron-chelated growth of *Y. pestis* strains in a $Ybt^- Yfe^-$ background. *Y. pestis* strains were grown in deferrated PMH2 medium without (A) or with (B) 45 μ M DIP at 37°C. The following strains were used: Yfe^- , KIM6-2031.1; $Yfe^- Yfu^-$, KIM6-2082.1; $Yfe^- Yfu^- Yiu^-$, KIM6-2123.2. An essential condition for demonstrating a differential growth effect by the *yfu* and *yiu* mutants was a low initial inoculum (OD_{620} of 0.005). The growth curves shown represent one of two independent experiments that yielded similar results.

ripheral routes of infection (32, 33, 35). The *Yfe* ABC transporter plays a role in the later stages of bubonic plague and transports iron, manganese, and possibly zinc. In vitro, growth defects due to mutation of the *Yfe* system are not readily detected except in a Ybt^- background (5, 25). Although the *Yfu* ABC transporter was proven functional as an iron uptake system in *E. coli*, we originally failed to find a phenotype for a $\Delta yfuA$ mutation in *Y. pestis* (25). In this study, we showed that the *Yfu* system functions in *Y. pestis* as an iron uptake system. However, a $Ybt^- Yfe^-$ background and DIP or EDDA iron chelators were required to demonstrate a phenotype for the Yfu^- mutant. Two plague hemin transport systems have been previously studied. The *Hmu* ABC transporter is required for the utilization of hemin and hemoproteins, while the *Has* system appears to be nonfunctional, at least under the in vitro conditions tested. Mutations in both systems did not affect the LD_{50} of these strains in a mouse model of bubonic plague (32, 35, 38, 47). Finally, genome sequence analysis has identified seven additional putative iron uptake systems (32, 35).

Here we characterized the *Yersinia* iron uptake ABC transporter, *YiuABC*, and a genetically linked gene encoding a putative TonB-dependent receptor, *YiuR*. This system is highly conserved in the sequenced *Y. pestis* genomes (including the three classical biotypes and the Pestoides group or *Microtus* biovar) and *Y. pseudotuberculosis* genomes (strains PB1 and IP32953). The unfinished genome sequence of *Y. enterocolitica*

apparently lacks *yiuA*, although it is possible that this gene will be present in the completed genome sequence. Outside of the *Yersinia* genus, *P. luminescens* subsp. *laumondii* contains ORFs with the highest similarities to the *Y. pestis* *YiuABC* system. *YiuR* has 63% similarity to the *V. cholerae* enterobactin receptor, *IrgA* (29). The *Yiu* ABC transporter has similarities to other ferric siderophore transport systems, with *YiuABC* ranging from 69% to 54% similar to the *Corynebacterium diphtheriae* *Irp6A-Irp6C* transporter that is essential for corynebactin-dependent iron uptake (37).

Our experimental results also indicate that the *Yiu* system functions in iron uptake. First, the expression of *yiuABC* in *E. coli* 1017, a strain defective in iron uptake, restored the ability of this strain to grow under iron-chelated conditions. Curiously, the *YiuR* OM receptor was not required for iron acquisition by the *YiuABC* transporter in *E. coli* 1017. Second, the *yiuA* promoter activity was repressed by iron via *Fur*. In addition, the *Y. pestis* Yiu^- strain exhibited growth defects when grown on plates containing a gradient of the iron chelator DIP or EDDA. The latter result suggests that *Yiu* can operate as an iron acquisition system in *Y. pestis*. However, the specific substrate for the *YiuABC* transporter is undetermined. Amino acid similarities suggest that this system may have an Fe-siderophore substrate. However, in these studies, we used the chemically defined medium, PMH or PMH2 (25, 45); thus, there should be no exogenous, contaminating siderophore

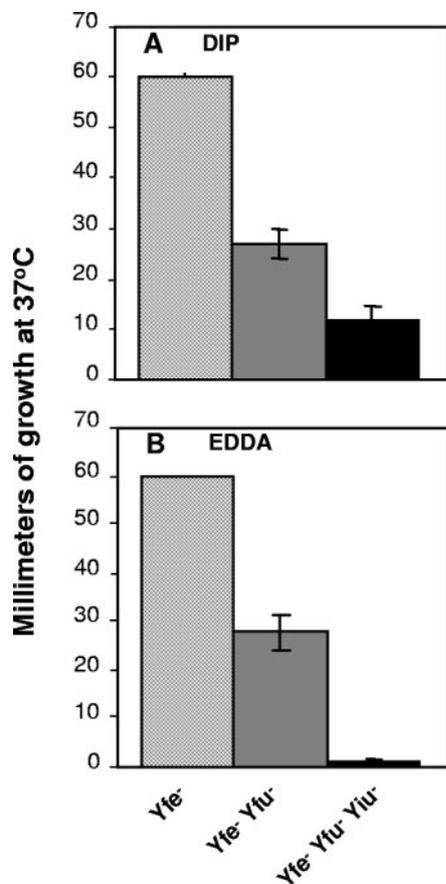


FIG. 5. Growth of Yfu⁻ and Yiu⁻ mutants across iron-chelator gradient plates. The growth of iron-depleted *Y. pestis* strains across PMH2 gradient plates containing DIP at 0 to 25 μ M (A) or EDDA at 0 to 1 μ M (B) at 37°C was monitored daily for 72 h. The growth distance was recorded from 0 mm (no growth) to 80 mm (confluent growth across the plate). The following strains were used: Yfe⁻, KIM6-2031.1; Yfe⁻ Yfu⁻, KIM6-2082.1; and Yfe⁻ Yfu⁻ Yiu⁻, KIM6-2123.2. The data shown are averages from two independent experiments. Error bars indicate standard deviations.

present in the growth media. While there are two other putative siderophore or nonribosomal peptide biosynthesis systems, Ysu and Ynp, encoded in the *Y. pestis* genome, their ability to synthesize a siderophore is questionable given IS insertions and possible frameshift mutations within genes of both systems. In addition, both systems have associated genes, encoding OM receptors and ABC transporters that would presumably function to accumulate any putative compound they might produce (32, 35). Alternatively, the Yiu system may weakly recognize ferric complexes with components of the defined medium (e.g., aromatic amino acids, vitamins, etc.).

Previous studies have indicated that some plague inorganic iron transport systems are more effective than others for in vitro and in vivo acquisition of iron. For example, growth defects due to a mutation in the Yfe transporter are readily apparent only in a Ybt⁻ background (5). In this study, we were able to demonstrate an iron-deficient growth defect in a $\Delta yfuA$ mutant, but only in a Ybt⁻ Yfe⁻ background (Fig. 4A). Finally, our $\Delta yiuABCR$ mutant also had an iron-chelated growth defect (Fig. 4B and 5), but again only when the Ybt, Yfe, and

Yfu iron transport systems were mutated. These results suggest a hierarchy of iron acquisition efficacy with Ybt > Yfe > Yfu > Yiu—at least for the four inorganic iron transporters that we have thus far characterized.

The mouse model of bubonic plague suggests an iron transport hierarchy similar to that defined by in vitro conditions. The Ybt system is absolutely essential for the virulence of plague from peripheral routes but is dispensable after the infection has reached the bloodstream (4, 5, 8, 20). The reason for the absolute requirement for this system from a subcutaneous route of infection is unknown. However, Ybt may be the only system effective under the environmental conditions and/or against the host chelators present in the lymphatics. It should be noted that the Ybt siderophore has higher binding affinity for ferric iron than does lactoferrin (33). The Yfe transporter has a modest effect on virulence from a subcutaneous route, while a Ybt⁻ Yfe⁻ double mutant is completely avirulent by an intravenous route (5, 25). Since the Yfe system is important in the later stages of bubonic plague, this system may be responsible for uptake of available iron in the spleen and liver, possibly from ferritin stores or other intracellular iron reservoirs. Previously, *Y. pestis* has been shown to acquire iron from ferritin in the absence of the Ybt system (42, 45). In contrast, the Yfu transporter had no apparent role in bubonic plague (25). Similarly, we found no evidence in this study for an in vivo role of the Yiu system, at least by a subcutaneous route of infection. These results suggest that Ybt and Yfe are the only effective iron acquisition systems in a mouse model of bubonic plague. Further studies will be required to determine whether any other iron transport systems play a role in pneumonic plague, in the infection of rodents other than mice, or even for growth in fleas.

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