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The Yersiniabactin Transport System Is Critical for the Pathogenesis of Bubonic and Pneumonic Plague▽

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Iron acquisition from the host is an important step in the pathogenic process. While *Yersinia pestis* has multiple iron transporters, the yersiniabactin (Ybt) siderophore-dependent system plays a major role in iron acquisition *in vitro* and *in vivo*. In this study, we determined that the Ybt system is required for the use of iron bound by transferrin and lactoferrin and examined the importance of the Ybt system for virulence in mouse models of bubonic and pneumonic plague. *Y. pestis* mutants unable to either transport Ybt or synthesize the siderophore were both essentially avirulent via subcutaneous injection (bubonic plague model). Surprisingly, via intranasal instillation (pneumonic plague model), we saw a difference in the virulence of Ybt biosynthetic and transport mutants. Ybt biosynthetic mutants displayed an ~24-fold-higher 50% lethal dose (LD_{50}) than transport mutants. In contrast, under iron-restricted conditions *in vitro*, a Ybt transport mutant had a more severe growth defect than the Ybt biosynthetic mutant. Finally, a Δ*pgm* mutant had a greater loss of virulence than the Ybt biosynthetic mutant, indicating that the 102-kb *pgm* locus encodes a virulence factor, in addition to Ybt, that plays a role in the pathogenesis of pneumonic plague.

Nearly all organisms require trace amounts of iron. Pathogens must overcome host iron- and heme-binding proteins to cause an infection and disease. The importance of iron acquisition mechanisms has been demonstrated in a number of bacterial pathogens (14, 15, 27, 32, 85). *Yersinia pestis*, the causative agent of plague, has a number of proven and putative iron and heme transport systems. Of these systems, the yersiniabactin (Ybt) siderophore-dependent iron transport system plays a major role in the virulence of bubonic plague in mice (7, 8, 38, 76, 79).

All identified genes required for the regulation, synthesis, and transport of Ybt, except for *ybtD*, are carried within a high-pathogenicity island (HPI) that has been spread among enteric pathogens but is essentially identical in the pathogenic yersiniae (13, 59, 79). In *Y. pestis*, the ~36-kb HPI is located within the 102-kb *pgm* locus; the entire *pgm* locus undergoes spontaneous deletion *in vitro* at a frequency of about 10^{-5} (25, 39, 62). The Ybt system produces a siderophore composed of one salicylate, one thiazoline, and two thiazolidine rings via a nonribosomal peptide/polyketide synthesis mechanism involving high-molecular-weight protein 1 (HMWP1), HMWP2, YbtD, YbtT, YbtE, YbtU, and YbtS (76, 79, 94). The formation constant of this siderophore with ferric iron is 4×10^{36}, and the crystal structure of the ferric complex has been solved (68, 78).

Iron from the Ybt-Fe complex is transported into the cell via the TonB-dependent outer membrane (OM) receptor Psn (which is also required for sensitivity to the bacteriocin pesticin) and an ABC transporter consisting of two inner membrane (IM), fused-function permease/ATP-binding proteins, YbtP and YbtQ. A mutation in any of these three genes prevents Ybt-dependent uptake of iron but does not prevent Ybt secretion. YbtX is encoded in an apparent four-gene operon (*ybtPQXS*) and is a predicted IM protein which resembles an exporter, with 12 predicted transmembrane domains (37, 76, 79, 80). It has weak homology to *Escherichia coli* EntS and *Bordetella* AlcS, exporters for enterobactin and alcaligin, respectively (18, 44, 83), but stronger similarities to RhtX and FptX, which import rhizobactin and pyochelin in *Sinorhizobium meliloti* and *Pseudomonas aeruginosa*, respectively (66, 70). A mutation in *ybtX* does not cause a significant defect in either Ybt synthesis or the ability to use Ybt as an iron source. Thus, the role of YbtX, if any, in the Ybt system remains an enigma (7, 37, 38, 76, 79).

In addition to typical Fur-Fe repression, maximal activation of the Ybt biosynthetic and transport operons requires an AraC-like positive regulator, YbtA, and its cognate siderophore, Ybt. Similar mechanisms activate diverse siderophore systems in a number of bacteria (21, 36, 47, 63, 65, 69, 76, 77, 79).

Here we show that the Ybt system can remove iron from transferrin and lactoferrin. In addition we examine the role of the Ybt system in the pathogenesis of plague in mice. Previously we showed that the Ybt system was required for virulence by a subcutaneous (s.c.) route of infection using an attenuated strain of *Y. pestis* (*yopJ psa*). Strains bearing mutations in the OM receptor (*psn*), the IM permease/ATPase (*ybtP*), or a Ybt biosynthetic enzyme (*irp2*) failed to kill mice at the highest doses tested (7, 37). We have obtained similar results in this study with a fully virulent strain. In addition, our experiments indicate that the Ybt system is also important in pneumonic plague. However, there were interesting differences in the virulence of siderophore receptor (*psn*) mutants and biosynthetic (*irp2*) mutants which were not seen in s.c. infections. Strains

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TABLE 1. *Y. pestis* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains[a]		
KIM5	Pgm$^-$ (Δ*pgm*; Hms$^-$ Ybt$^-$) Lcr$^+$ Pla$^+$; pMT1, pCD1, pPCP1	92
KIM5(pCD1Ap)+	Ap^r Pgm$^+$ (Hms$^+$ Ybt$^+$) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6+	45
KIM5(pCD1Ap)	Ap^r Pgm$^-$ (Δ*pgm*; Hms$^-$ Ybt$^-$) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6	This study
KIM5-2045.1 (pCD1Ap)	Ap^r Hms$^+$ Ybt$^-$ (Δ*psn2045.1*) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6-2045.1	This study
KIM5-2045.6 (pCD1Ap)	Km^r Ap^r Hms$^+$ Ybt$^-$ (Δ*psn*::*kan2045.6*) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6-2045.6	This study
KIM5-2046.1 (pCD1Ap)	Km^r Ap^r Hms$^+$ Ybt$^-$ (*irp2*::*kan2046.1*) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6-2046.1	This study
KIM5-2046.3 (pCD1Ap)	Ap^r Hms$^+$ Ybt$^-$ (Δ*irp2-2046.3*) Lcr$^+$ Pla$^+$; pMT1, pCD1Ap (*'yadA*::*bla*), pPCP1; derived from KIM6-2046.3	This study
KIM6+	Pgm$^+$ (Hms$^+$ Ybt$^+$) Lcr$^-$ Pla$^+$; pMT1, pPCP1	38
KIM6	Pgm$^-$ (Δ*pgm*; Hms$^-$ Ybt$^-$) Lcr$^-$ Pla$^+$; pMT1, pPCP1	38
KIM6-2045.1	Hms$^+$ Ybt$^-$ (Δ*psn2045.1*) Lcr$^-$ Pla$^+$; pMT1, pPCP1	38
KIM6-2045.6	Km^r Hms$^+$ Ybt$^-$ (Δ*psn*::*kan2045.6*) Lcr$^-$ Pla$^+$; pMT1, pPCP1	36
KIM6-2046.1	Km^r Hms$^+$ Ybt$^-$ (*irp2*::*kan2046.1*) Lcr$^-$ Pla$^+$; pMT1, pPCP1	38
KIM6-2046.3	Hms$^+$ Ybt$^-$ (Δ*irp2-2046.3*) Lcr$^-$ Pla$^+$; pMT1, pPCP1	7
KIM6-2180	Hms$^+$ Ybt$^-$ (Δ*irp2-2046.3* Δ*psn2045*.1) Lcr$^-$ Pla$^+$; pMT1, pPCP1	This study
Plasmids		
pCD1Ap	71.7 kb, Ap^r Lcr$^+$; pCD1 with *bla* cassette inserted into *'yadA* downstream of the frameshift mutation in this pseudogene	45
pCSIRP498.9	8.7 kb, Ap^r Suc^s (*sacB*$^+$), Δ*irp2-2046.3*, R6K origin suicide vector	7

[a] A plus sign indicates an intact chromosomal 102-kb *pgm* locus. All other *Y. pestis* strains have a mutation within this locus or a deletion of the entire locus.

which produce the siderophore but are unable to use it (i.e., *psn* mutants) were more virulent than the biosynthetic mutants. However, *in vitro* the *psn* mutant was more defective than the biosynthetic mutant for growth under iron-restricted conditions.

MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains and plasmids used in this study are listed in Table 1. From glycerol stocks (10), *Y. pestis* strains were grown on Congo red (CR) agar (88) before being transferred to tryptose blood agar base (TBA) slants. Formation of red colonies on CR plates indicates that the strain has retained the *pgm* locus, which can be spontaneously lost at a rate of 10^{-5} (25, 39, 62).

For iron-deficient growth studies, *Y. pestis* cells were harvested from TBA slants and grown in chemically defined medium (PMH or PMH2) which had been extracted prior to use with Chelex 100 resin (Bio-Rad Laboratories). A previously published paper by Gong et al. has an error in the published buffer concentrations; the concentrations of PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] and HEPES should be 50 mM for PMH2 and PMH, respectively (45, 86). For iron-replete growth, *Y. pestis* strains were cultivated in PMH or PMH2 supplemented with 10 μM $FeCl_3$. Growth of the cultures was monitored by determining the optical density at 620 nm (OD_{620}) with a Genesys5 spectrophotometer (Spectronic Instruments, Inc.). Growth through two transfers (~6 to 8 generations) was used to acclimate cells to PMH2 and varying iron conditions prior to use in experimental studies.

All glassware used for iron-restricted studies was soaked overnight in Scot-Clean (OWL Scientific, Inc.) to remove contaminating iron and copiously rinsed in deionized water. Where appropriate, ampicillin (Ap) (50 to 100 μg/ml) or kanamycin (Km) (50 μg/ml) was added to media.

Construction of *Y. pestis* KIM6-2180 (Δ*psn2045.1* Δ*irp2-2046.3*). Suicide plasmid pCSIRP498.9 (encoding an in-frame Δ*irp2-2046.3* mutation) was electroporated into *Y. pestis* KIM6-2045.1 (Δ*psn2045.1*), and merodiploids were selected on TBA plates containing Ap. As previously described (8), selected cointegrants were grown overnight in heart infusion broth (HIB) without Ap and plated on CR plates containing 5% sucrose to select for recombinants with the Δ*irp2-2046.3* mutation. The mutation was confirmed by Southern blot analysis (data not shown), and the Δ*irp2-2046.3* Δ*psn2045.1* double mutant was designated *Y. pestis* strain KIM6-2180 (Table 1).

Plasmids and DNA techniques. Plasmids were purified by alkaline lysis from cultures grown overnight in HIB (12). *Y. pestis* cells were transformed by electroporation as previously described (38).

Assay for use of Tf and Lf. *Y. pestis* strains KIM6+ (Pgm$^+$) and KIM6-2046.1 (*irp2*::*kan2046.*1) were grown through two transfers at 37°C in deferrated PMH with or without 0.5 mM $NaHCO_3$ (PMH-$NaHCO_3$). Second-transfer overnight cultures were used to seed 20 ml of molten PMH-$NaHCO_3$–1% agarose with ~5×10^6 cells, and ethylenediamine-di(*o*-hydroxyphenyl-acetic acid) (EDDA) was added to a final concentration of 15 μM or 7.5 μM to inhibit the growth of KIM6+ or KIM6-2046.1 (*irp2*::*kan2046.1*), respectively, on plates for transferrin (Tf) growth responses. For tests of lactoferrin (Lf) growth responses, 50 μM EDDA was used to inhibit the growth of KIM6+ and KIM6-2046.3 (Δ*irp2-2046.3*). Aliquots of 15 μl of various iron sources were placed on the plates: partially iron-saturated Tf (Sigma; 50 mg/ml) or 1 mM $FeCl_3$ was added to 1-mm-diameter wells in the agar, while bovine Lf (Sigma; 50 mg/ml) or 1 mM $FeSO_4$ was placed on filter discs. Alternatively, PMH-$NaHCO_3$-EDDA plates seeded with *Y. pestis* strains were overlaid with a dialysis membrane (12,000- to 14,000-Da molecular mass cutoff), and mixtures of PMH, 2% agarose, and the above-described iron-containing preparations were placed as a drop on the dialysis membrane. Although no information on the degree of iron saturation of bovine Lf is provided by Sigma, it is ~15 to 20% saturated in its natural state (87). To remove free iron, Lf resuspended in a dialysis buffer (0.1 M sodium citrate–0.1 M sodium bicarbonate, pH 7.2) with 0.4% sodium azide at 150 mg/ml was dialyzed (12,000- to 14,000-Da molecular mass cutoff) against 500 ml of this buffer containing 0.4% sodium azide and 20 μM EDDA for 40 min with two buffer changes. Following overnight dialysis at 4°C in the buffer without EDDA or sodium azide, the Lf solution was adjusted to 50 mg/ml in PMH2 containing 2 mM sodium bicarbonate. Bacterial growth on the plates was monitored over 3 days for Tf and 2 days for Lf and visualized by overlayering the plate with TBA containing 2 mM ferric citrate and 1.5 mM esculin. *Y. pestis* hydrolyzes esculin to produce a black precipitate in areas of growth.

Urea gel electrophoresis of iron-Tf complexes. *Y. pestis* strains KIM6+ and KIM6-2046.1 were grown at 37°C through three transfers in iron-depleted PMH-$NaHCO_3$. Dialysis baggies (12,000- to 14,000-Da molecular mass cutoff) containing iron-saturated Tf at a concentration of 1 mg/ml in PMH2 were placed in a suspension of *Y. pestis* cells in PMH-$NaHCO_3$ or in uninoculated medium as a

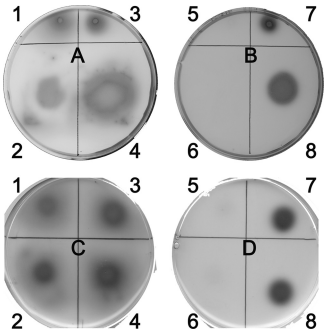

FIG. 1. Use of iron from transferrin (Tf) and lactoferrin (Lf). Growth responses of Ybt$^+$ KIM6+ (A and C), Ybt$^-$ KIM6-2046.1 (*irp2*::*kan2046.1*) (B), and KIM6-2046.3 (Δ*irp2-2046.3*) (D) on PMH-EDDA plates to partially saturated Tf (1, 2, 5, and 6 in panels A and B), partially saturated bovine Lf (1, 2, 5, and 6 in panels C and D), or inorganic iron (3, 4, 7, and 8) are shown. The solutions were added to wells (A and B) or on filter discs (C and D) on seeded plates (1, 3, 5, and 7) or spotted onto a dialysis membrane overlaying the bacterial cells (2, 4, 6, and 8). After incubation with Tf or Lf at 37°C, plates were overlaid with TBA containing esculin and ferric citrate to visualize bacterial growth. The images are from one of two or more independent experiments that yielded similar results.

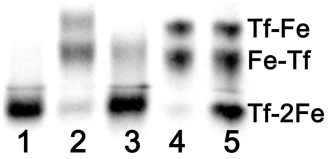

FIG. 2. Removal of iron from iron-saturated human transferrin by *Y. pestis*. Tf was incubated overnight with iron-starved cultures of *Y. pestis* Ybt$^+$ KIM6+ (lane 2) or Ybt$^-$ KIM6-2046.1 (lane 3). Tf was in a dialysis baggy (12,000- to 14,000-Da molecular mass cutoff), separating it from the cultures. After incubation, the various iron-saturated Tf forms were separated on 6% polyacrylamide-urea gels. Incubation of Tf with PMH growth medium (lane 1) was used as a negative control. Partially saturated Tf (lane 4) and a mixture of various forms of Tf (lane 5) were used to show mobility shifts due to iron saturation of Tf. The image is from one of two independent experiments, which yielded similar results.

negative control. After overnight incubation at 37°C, a 10-μl aliquot of the Tf samples was mixed with sample buffer and electrophoresed through a 6.5 M urea–6% polyacrylamide gel in Tris-borate buffer (pH 8.3) as previously described (33, 64). To identify changes in transferrin saturation, partially saturated Tf (a mixture of N- and C-end iron-loaded monosaturated forms) (Sigma) was electrophoresed, as well as an equimolar mixture of holo-Tf (fully iron saturated) (ICN Biomedicals Inc.). The proteins were visualized using Coomassie blue staining.

Virulence testing. Construction of potentially virulent strains and virulence testing were performed in a CDC-approved biosafety level 3 (BSL3) laboratory following select agent regulations, using procedures approved by the University of Kentucky Institutional Biosafety Committee. *Y. pestis* strains were transformed with the virulence plasmid pCD1Ap by electroporation (43, 45) and plated on TBA plates containing Ap (50 μg/ml). The plasmid profile of transformants was analyzed, as well as their phenotype on CR agar (88) and magnesium-oxalate plates (48). Supernatants from cultures grown at 37°C in the absence of $CaCl_2$ were tested for the secretion of LcrV by Western blot analysis using polyclonal antisera against histidine-tagged LcrV (40, 43).

For s.c. infections, overnight cultures of *Y. pestis* cells grown in HIB at 26°C were diluted to an OD of 0.1 at 620 nm and incubated in HIB at 26°C until they reached mid-logarithmic phase (OD of ~0.5). Samples were harvested and diluted in mouse isotonic PBS (149 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4). Groups of four 6- to 8-week-old female Swiss Webster (Hsd::ND4) mice were injected subcutaneously with 0.1 ml of 10-fold serially diluted bacterial suspensions ranging from 100 to 10^8 CFU/ml.

Cells used for intranasal (i.n.) instillation infections were grown at 37°C in HIB containing 4 mM $CaCl_2$ to prevent full induction of Lcr *in vitro* and were similarly diluted in mouse isotonic PBS. Twenty microliters of the bacterial suspension was administered to the nares (~5-μl doses alternating between the two nostrils) of mice sedated with 100 μg of ketamine and 10 μg of xylazine per kg of body weight. The actual i.n. and s.c. bacterial doses administered were determined by plating aliquots of serially diluted suspensions of each dose, in duplicate, onto TBA plates containing Ap (50 μg/ml). The colonies were counted on plates incubated at 30°C for 2 days. Mice were observed daily for 2 weeks, and 50% lethal doses (LD_{50}s) were calculated according to the method of Reed and Muench (84). All animal care and experimental procedures were conducted in accordance with the Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (69b), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (69a), and the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Teaching, Research, and Training* (70a) and were approved by the University of Kentucky Institutional Animal Care and Use Committee. The University of Kentucky Animal Care Program first obtained accreditation from the Association for the Assessment and Accreditation of Laboratory Animal Care, Inc. (AAALAC), in 1966 and has maintained full accreditation continuously since that time.

RESULTS

Ybt-dependent use of host iron-binding proteins. We tested iron-stressed cells of *Y. pestis* KIM6+ (Ybt$^+$), KIM6-2046.1 (*irp2*::*kan2046.1*; Ybt$^-$), or KIM6-2046.3 (in-frame Δ*irp2-2046.3*; Ybt$^-$) for their ability to use Tf and Lf as sole sources of iron for growth on PMH-$NaHCO_3$-EDDA plates. A functional Ybt system allowed the use of both host proteins and inorganic iron even when the cells were separated from the solutions by a dialysis membrane. Inorganic iron stimulated the growth of the Ybt$^-$ mutant. The Ybt$^-$ mutant was unable to use either Tf or Lf as an iron source (Fig. 1).

We also demonstrated the ability of Ybt to remove iron from Tf. The mobility of Tf in polyacrylamide gels containing urea is affected by iron saturation and which of the two iron-binding sites are filled (33, 41, 50, 64, 96). Iron-saturated Tf was separated from cultures by a dialysis baggy and incubated overnight at 37°C. Subsequent urea gel electrophoresis of the Tf solution showed that incubation with a KIM6+ culture converted the majority of Fe-saturated Tf to a less saturated form (Fig. 2, lane 2). In contrast, the *Y. pestis irp2* mutant was able to convert only a small amount of saturated Tf to an Fe-Tf form; this conversion was not seen in uninoculated medium (Fig. 2, compare lanes 1 lane 3). Nevertheless, these results indicate that Ybt is able to directly remove iron from Tf as well as use iron from Lf.

Ybt and bubonic plague. Previously we tested the virulence of various iron transport mutants using mildly attenuated strains of *Y. pestis* with mutations in *yopJ* and *psa*. In this study we used a reconstructed wild-type (WT) strain [KIM5 (pCD1Ap)+] to test the effects of Ybt transport and biosynthesis mutations on virulence. Table 2 shows the LD_{50}s in mice infected by an s.c. route. Twenty-five cells killed 50% of mice infected with this WT strain; with doses greater than the LD_{50}, mice had ruffled fur starting on day 3 postinfection and developed a hunched posture by day 5, with deaths occurring between days 5 and 13. In contrast, doses of ~10^7 for both the Ybt transport and biosynthesis mutants caused transient illness (ruffled fur and hunched posture at the highest doses), but only 1 of 16 mice infected with the Δ*irp2* biosynthetic mutant died

TABLE 2. LD_{50}s for *Y. pestis* strains in mouse models of pneumonic and bubonic plague

Model	Strain or mutation	LD_{50} (mean ± SD)[a]
Pneumonic plague	Wild type	329 ± 105
	Δ*psn2045.1* or *psn*::*kan2045.6*	$1.1 \times 10^4 \pm 2.9 \times 10^3$
	Δ*irp2-2046.3* or *irp2*::*kan2046.1*	$2.6 \times 10^5 \pm 1.8 \times 10^5$
	Δ*pgm*[b]	$>3.9 \times 10^6$
Bubonic plague	Wild type	25 ± 12
	Δ*psn2045.1* or *psn*::*kan2045.6*	$>2.6 \times 10^7$
	Δ*irp2-2046.3* or *irp2*::*kan2046.1*	$>1.3 \times 10^7$

[a] > indicates an LD_{50} above the highest bacterial doses tested from at least two independent experiments. Values were calculated from two or more independent trials. Probit analysis using SPSS determined that the intranasal LD_{50}s of the three mutants are significantly different from each other (see text). For statistical analysis, the Δ*pgm* LD_{50} was set at 3.9×10^6.

[b] Two Δ*pgm* strains [KIM5 and KIM5(pCD1Ap) (Table 1)] with a native and recombinant pCD1 were tested but showed the same loss of virulence.

(day 5 postinfection). Note that two independent mutations in *psn* and *irp2* yielded similar results, indicating that the loss of virulence is not due to an unidentified secondary mutation in these strains. Consequently, the Ybt system is critical for virulence by this route of infection, with mutants displaying a $>4.3 \times 10^5$-fold loss of virulence in this model.

Ybt and pneumonic plague. An i.n. route has been used to initiate pneumonic infections by *Brucella melitensis*, *Chlamydia trachomatis*, *Francisella tularensis*, *P. aeruginosa*, *Streptococcus pneumoniae*, and *Y. pestis* (3, 4, 28, 32, 51, 54, 56, 72, 81, 90, 97). Latham et al. (54) noted that the i.n. model in mice causes a severe bronchopneumonia that closely resembles descriptions of the disease in humans and nonhuman primates. The Lyons research group has followed bacterial organ loads, disease time course, and pathology and found that i.n. infection is a valid pneumonic plague model in mice (C. R. Lyons, personal communication). Lathem et al. (54) calculated an i.n. LD_{50} for strain CO92 of 260 cells, with KIM5(pCD1Ap)+ giving a similar value. Our KIM5(pCD1Ap)+ strain yielded an i.n. instillation LD_{50} of 329 ± 105. At doses above the LD_{50}, mice exhibited ruffled fur by day 2 postinfection, with deaths occurring between days 3 and 5. In contrast, our Δ*psn* and Δ*irp2* mutants yielded LD_{50}s of approximately 10^4 and 10^5. Again we used two independent *psn* and *irp2* mutations to ensure that the loss of virulence was not due to a secondary mutation (Table 2). While both the *psn* and *irp2* mutants were significantly less virulent than WT, the difference between the two mutants was unexpected and intriguing. Table 2 shows the averaged LD_{50}s for the strains unable to produce the Ybt siderophore (*irp2* mutants) and those able to synthesize Ybt but unable to utilize it (*psn* mutants). An ~24-fold difference ($P = 0.0076$) was maintained between the biosynthetic and transport mutants, with the *psn* mutants being 33-fold less virulent and the *irp2* mutants being 790-fold less virulent than the WT. These results were surprising since *in vitro*, a biosynthetic mutant has less of an iron uptake defect than a mutant which produces but cannot utilize the Ybt siderophore (78). A time-to-death analysis of mice infected with the Ybt^+ strain and the *irp2* and *psn* mutants is shown in Fig. 3. Mice infected with the Ybt^+ strain (average dose of 1,153 cells) died within 3 to 4 days postinfection. Higher average doses of the *psn* and *irp2* mutants (17,050 and 268,000 cells, respectively) were lethal, beginning at 5 to 6 days postinfection and lasting until day 8.

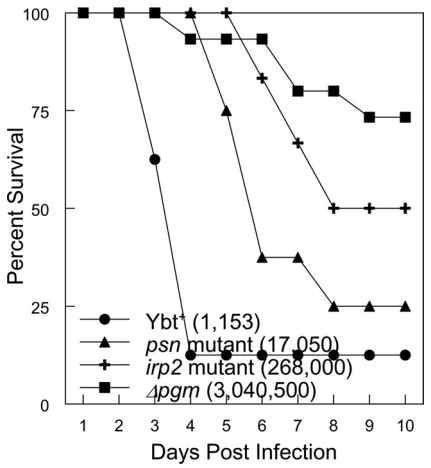

FIG. 3. Time-to-death analysis from i.n. instillation studies. Except for the Δ*pgm* mutant, infectious doses used were close to the calculated LD_{50} for that strain. The average doses (in parentheses) were calculated from two (Ybt^+ and *psn*), three (*irp2*), and four (Δ*pgm*) independent experiments. All studies were carried out to 14 days, with no further deaths after day 10. Data are averages from all LD_{50} studies, shown as percent survival on the indicated days postinfection.

We previously hypothesized that the Ybt secreted by the transport mutant chelates residual iron in iron-deficient media, making it unavailable to other *Y. pestis* iron transport systems (78). Hence, these mutants would have more of a growth defect in iron-deficient media than strains that do not produce the siderophore. The growth patterns of biosynthetic and transport mutants of the Ybt system under iron-restrictive conditions are shown in Fig. 4. The transport (*psn*) mutant exhibits a significant growth defect compared to the WT strain and the

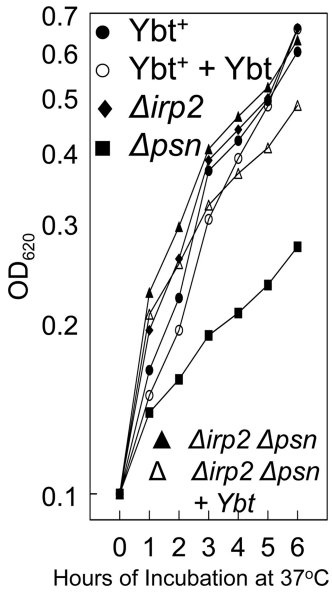

FIG. 4. Iron-deficient growth of *Y. pestis* strains. All strains were grown in deferrated PMH2 at 37°C. Where indicated, purified Ybt was added to KIM6+ (Ybt^+) and KIM6-2180 (Δ*irp2-2046.3* Δ*psn2045.1*) at a concentration similar to that produced by a Ybt^+ strain. The growth curves shown are from one of two independent experiments, which yielded similar results.

biosynthetic mutant (Fig. 4). This growth defect is relieved in an *irp2 psn* mutant, which can neither synthesize nor use Ybt. Addition of the Ybt siderophore to the growth medium retards the growth of the double mutant (Fig. 4). Note that this growth defect is not as severe as observed with the *psn* mutant. This is likely due to the high concentration of Ybt which is produced by the *psn* mutant throughout the growth of the cells in deferrated PMH2 (three transfers); Ybt was added to the *irp2 psn* double mutant only during the third transfer shown in Fig. 4. Thus, these results indicate that, *in vitro*, Ybt production in a Ybt transport mutant is detrimental to iron-restricted growth.

The *pgm* locus and plague. Early studies showed that Pgm^- (putative or proven Δ*pgm*) mutants were avirulent by peripheral routes of infection but fully virulent if injected intravenously (26, 52, 92). Specific *ybt* mutations within the *pgm* locus clearly demonstrate that the Ybt system is essential for bubonic plague and of critical importance for pneumonic plague (Table 2). We also tested two independent Δ*pgm* mutants by an i.n. instillation route of infection. Both mutants yielded similar results; overall, 73.3% (11/15) of the mice survived administration of $\sim 3 \times 10^6$ cells. Thus, the LD_{50} of a Δ*pgm* mutant is >11,850- and >15-fold higher than those of the WT and the *irp2* mutant ($P = 0.0013$), respectively. At the highest dose, the Δ*pgm* mutant killed fewer mice than the *irp2* mutant and had a time-to-death range that was slightly delayed compared to that of the *psn* mutant (Fig. 3). If the avirulence of the Δ*pgm* strain was strictly due to the absence of the Ybt iron transport system, then we would have expected it to have an LD_{50} and a time-to-death range similar to those of the *irp2* mutant. These data clearly suggest that an additional factor or factors encoded within the *pgm* locus play a role in pneumonic plague.

DISCUSSION

Use of host iron sources by Ybt. Our *in vitro* analyses have demonstrated that the Ybt siderophore can remove iron from Tf and have suggested that it can remove iron from Lf. A Ybt^+ strain was capable of using these compounds as iron sources for growth when separated from the compounds by a dialysis baggy. Thus, a secreted diffusible molecule seems to be required for this growth response. We used gel electrophoresis to demonstrate that Ybt was involved in removing iron from Tf. In contrast, a Ybt biosynthetic mutant failed to respond to these iron sources when separated by a dialysis membrane, supporting the conclusion that the Ybt siderophore is required to use the bound iron under these conditions. The Tf and Lf results were not unexpected, since the *Y. pestis* KIM10+ genome (derived from KIM6+) encodes no apparent OM receptors for these compounds. Since we used iron chelators to prevent growth of *Y. pestis* strains without added host iron sources, it remains a formal possibility that the Ybt siderophore removed iron chelated by EDDA rather than directly from Lf.

Ybt and virulence. Our previous LD_{50} studies of bubonic plague in mice used bacterial strains with background mutations in *yopJ* and *psa* that caused a slight attenuation compared to a fully virulent WT background (~5-fold loss of virulence) (7, 8). To assess whether the *yopJ psa* background artificially enhanced the virulence defect due to *ybt* mutations, we tested both Ybt biosynthetic and transport mutants in an otherwise WT background. We also tested higher bacterial doses than in previous studies. Our results indicate that loss of the Ybt system causes a $>4.3 \times 10^5$-fold loss of virulence by an s.c. route of infection. The Ybt biosynthetic and transport mutants showed similar decreases in virulence by this route of infection.

In other bacteria, siderophores as well as heme transport systems have been implicated in iron acquisition in the lung. For example, legiobactin, ornibactin, and alcaligin are required for lung infections by *Legionella pneumophila*, *Burkholderia cenocepacia*, and *Bordetella pertussis*, respectively (2, 19, 93). *B. pertussis* also uses enterobactin and a heme uptake system to acquire iron during lung infections (20, 22). In *Klebsiella pneumoniae*, Ybt played a major role in iron acquisition in the lung (55).

Two different mutations in *psn* (encoding the OM receptor for Ybt) caused an ~33-fold loss of virulence. In contrast to our results in the bubonic model, two different *irp2* mutants that are unable to produce the Ybt siderophore caused an even greater loss of virulence than the *psn* mutant, which can produce Ybt but is unable to use it: an ~24-fold greater loss than with the *psn* mutants and a 790-fold loss of virulence compared to the parental WT strain (Table 2). Since the Ybt biosynthetic mutant was not completely avirulent, one of the other *Y. pestis* iron transport systems may be modestly effective in acquiring iron during a lung infection.

The difference in virulence between the *psn* and *irp2* mutants is intriguing, especially since transport mutants are more detrimental to *in vitro* iron-restricted growth and iron uptake than biosynthetic mutants (78). This is the opposite of the results we found in the pneumonic plague model (i.e., the strain showing the most *in vitro* growth defect was more virulent). In the lungs, the Ybt siderophore may have other effects in addition to its role in providing iron. Although it is clear that Ybt serves as a signal molecule to activate transcription from *ybt* promoters (5, 36, 74, 77), the Ybt/YbtA signaling pathway in *Y. pestis* has not been entirely elucidated. We have favored a model in which the Ybt siderophore is transported into the cell and interacts with YbtA to transcriptionally activate regulated genes. There is good evidence for this type of regulation in *Pseudomonas aeruginosa* and *Bordetella*, which have AraC family regulators that respond to their cognate siderophores (17, 21, 67). While TonB-dependent signaling through the OM receptors of some other bacterial iron transport systems has been demonstrated (16), uptake mutants (*psn*, *tonB*, *ybtP*, and/or *ybtQ*) all show normal *ybt* gene regulation (7, 77, 80). The Ybt siderophore is a potent signaling molecule; growth stimulation by Ybt requires concentrations ~500-fold higher than the concentration needed to activate transcription of the *ybtP* promoter (77). Consequently, we propose that small amounts of Ybt, sufficient to serve its signaling function, enter the cell via alternate routes. Since our *in vitro* studies indicate that a *psn* mutant can sense and respond to the Ybt siderophore, perhaps these small amounts are sufficient to activate transcription of other virulence factors in *Y. pestis*. In *P. aeruginosa*, the siderophore pyoverdine regulates not only its own production but also that of additional virulence determinants (9, 53).

An alternative to the signaling hypothesis is that the siderophore affects the host environment or innate immunity. Deferrated hydroxamate siderophores (desferrioxamine, desferrichrome, and desferriaerobactin) have an immunosuppres-

sive effect on isolated mouse spleen mononuclear cells. In addition, enterobactin, independent of iron chelation, and desferrioxamine are cytotoxic for proliferating T cells (6, 49). However, other groups using desferrioxamine have found stimulatory effects on inflammatory cytokine production by intestinal and U937 cell lines (29, 57, 91). Pyochelin, which resembles Ybt structurally, can generate hydroxyl radicals and, under the appropriate conditions, damage pulmonary endothelial and epithelial cells (23, 24, 30). In addition, desferrithiocin, an iron chelator that is structurally similar to pyochelin, has been shown to inhibit T-cell proliferation (11). Recently, purified Ybt was shown to reduce the generation of reactive oxygen species by polymorphonuclear leukocytes (PMNs), human monocytes, and J774A.1 cells *in vitro* (71). Thus, Ybt may have direct toxic effects, affect host immune cell recruitment, and/or affect the synthesis of proinflammatory cytokines and/or reactive oxygen species.

The *pgm* locus and virulence. Another unexpected finding of this study was that the Δ*pgm* mutant showed an even greater loss of virulence than the *irp2* mutants (>15-fold), a >11,800-fold loss of virulence compared to the WT strain (Table 2). Other investigators have examined the virulence of Δ*pgm* strains via a pneumonic route of infection (73, 95). The LD_{50}s obtained range from 10^4 to ~10^6 cells. The differences in the observed LD_{50}s could be the result of a number of factors, including the strain of mouse used, the method of administering the bacteria, and the way that the bacterial cells were grown. None of the other groups compared the virulence of a Δ*pgm* strain with that of an Ybt mutant. Lee-Lewis and Anderson (58) did find that intraperitoneal administration of iron increased the virulence of a Δ*pgm* strain but not to the level of a wild-type strain. Interestingly, this group also demonstrated that the Δ*pgm* mutant did not cause pneumonic disease. The authors concluded that additional factors within the *pgm* locus play a role in pneumonic plague. Our results also clearly suggest that the 102-kb *pgm* locus, which includes the Ybt high-pathogenicity island and *hms* biofilm genes, encodes one or more virulence factors in addition to the Ybt system that play a role in pneumonic plague.

The only genes carried within the *pgm* locus for which there are published virulence studies are *hmsR* and *hmsH*; both are required for biofilm development (42, 60, 61, 75). An *hmsR* mutant was tested in a bubonic plague model, while the *hmsH* mutant was tested for virulence in mice via subcutaneous (bubonic plague) and intranasal (pneumonic plague) routes of infection. These studies found no significant role of biofilm formation in the virulence of either form of plague (1, 60).

The *ripABC* locus, also carried within the *pgm* locus, is required for survival in macrophages activated after bacterial infection (82). In collaboration with Jim Bliska's research group, we have found that the Δ*ripABC* mutant was fully virulent in our mouse model of pneumonic plague (J. Bliska and R. D. Perry, unpublished observations). However, a number of other open reading frames (ORFs) that could have effects on virulence are carried within the remaining >60 kb of the *pgm* locus. There are loci potentially encoding a pilus, a ferrous transporter distantly related to the Ftr/Efe family and more closely related to a newly identified FetMP ferrous transporter (36, 47, 52a, 89), two cation transporters, and six transcriptional regulators. Extensive experimental analysis will be required to determine if one or more of these loci are involved in the further loss of virulence of the KIM Δ*pgm* mutant (34) in our mouse model of pneumonic plague.

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