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Yersinia ironomics: comparison of iron transporters among *Yersinia pestis* biotypes and its nearest neighbor, *Yersinia pseudotuberculosis*

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Abstract Although *Yersinia pestis* epidemic biovars and *Yersinia pseudotuberculosis* are recently diverged, highly related species, they cause different diseases via disparate transmission routes. Since iron transport systems are important for iron acquisition from hosts and for survival in the environment, we have analyzed potential iron transport systems encoded by epidemic and non-epidemic or endemic strains of *Y. pestis* as well as two virulent *Y. pseudotuberculosis* strains. Computational biology analysis of these genomes showed a high degree of identity/similarity among 16 proven or possible iron/heme transporters identified. Of these, 7 systems were essentially the same in all seven genomes analyzed. The remaining 9 loci had 2–6 genetic variations among these genomes. Two untested, potential siderophore-dependent systems appear intact in *Y. pseudotuberculosis* but are disrupted or absent in all the endemic *Y. pestis* strains as well as the epidemic strains from the *antiqua* and *mediaevalis* biovars. Only one of these two loci are obviously disrupted in *Y. pestis* CO92 (epidemic *orientalis* biovar). Experimental studies failed to identify a role for hemin uptake systems in the virulence of pneumonic plague and suggest that *Y. pestis* CO92 does not make a siderophore other than Ybt.

Keywords Iron transport · Plague · Siderophore · Heme transport · *Yersinia pestis* · *Yersinia pseudotuberculosis* · Yersiniabactin

S. Forman and J.T. Paulley contributed equally to this study and are co-first authors.

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Introduction

The genus *Yersinia* includes environmental species, a fish pathogen, two enteropathogens (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) and *Yersinia pestis.* While the three epidemic biotypes/biovars of *Y. pestis* (*antiqua, mediaevalis*, and *orientalis*) cause bubonic, septicemic, and pneumonic plague, two other related groups (Microtus and Pestoides; endemic strains) are avirulent or highly attenuated in humans but cause a bubonic plague-like illness in some animals. These endemic strains have phenotypic characteristics which are intermediate between the nearest neighbor, *Y. pseudotuberculosis*, and the highly

pathogenic epidemic strains of *Y. pestis*. *Y. pestis* is thought to have diverged from *Y. pseudotuberculosis* anywhere from 1,500 to 20,000 years ago and the two species show ~97% gene homology and co-linear gene organization (Achtman et al. 2004; Anisimov et al. 2004; Chain et al. 2004; Hinchliffe et al. 2003; Zhou et al. 2004b). Despite this, the two species have distinct transmission routes (fecal-oral vs. flea) and cause completely different diseases. *Y. pestis* causes highly fatal acute diseases—bubonic, septicemic, and pneumonic plague—while *Y. pseudotuberculosis* causes a mesenteric lymphadenitis that is generally self-limiting. Although *Y. enterocolitica* causes similar but less severe disease pathologies, it diverged from *Y. pseudotuberculosis* approximately 200 million years ago. Consequently, *Y. enterocolitica* is significantly more divergent from *Y. pestis* and *Y. pseudotuberculosis* (Anisimov et al. 2004; Brubaker 2004; Perry and Fetherston 1997; Straley and Starnbach 2000; Thomson et al. 2006; Wren 2003). While a few genes important for colonization of and survival in fleas have been identified, genomic and experimental analysis has failed to clearly identify unique *Y. pestis* genes responsible for the switch from a mild chronic disease caused by *Y. pseudotuberculosis* to an acute lethal disease (Brubaker 2004; Chain et al. 2004; Hinchliffe et al. 2003; Hinnebusch 2004; Lorange et al. 2005; Zhou et al. 2004a).

Iron acquisition from the host is one key virulence determinant for most pathogens. Indeed many bacteria encode multiple iron and heme uptake systems (Byers and Arceneaux 1998; Crosa et al. 2004; Guerinot 1994; Mietzner and Morse 1994; Schaible and Kaufmann 2004; Weinberg and Weinberg 1995). In *Y. pestis,* only two iron transport systems have been shown to be important for the progression of bubonic plague—the yersiniabactin (Ybt) siderophore-dependent system and Yfe which transports iron and manganese (Bearden et al. 1997; Bearden and Perry 1999; Fetherston et al. 1999; Gong et al. 2001; Kirillina et al. 2006; Perry 2004; Perry and Fetherston 2004; Rossi et al. 2001). Given the importance of iron acquisition, it is possible that differences in iron uptake abilities may help account for the variation in host susceptibilities and types of disease among epidemic and endemic *Y. pestis* strains as well as its nearest neighbor *Y. pseudotuberculosis*. We have not analyzed *Y. enterocolitica* iron transport systems since it is more distantly related to the other two mammalian pathogens. A more general comparison of iron transport systems that includes *Y. enterocolitica* has been published (Perry 2004; Perry and Fetherston 2004) We have used computational analysis to assess iron transport systems in the genomes of five *Y. pestis* strains and two *Y. pseudotuberculosis* strains.

Methods and materials

Computational analyses

Yersinia pestis strains KIM10+, CO92, Antiqua, Pestoides F, and 91001 as well as *Y. pseudotuberculosis* strains IP32953 and PB1/+ have been sequenced (Chain et al. 2004, 2006; Deng et al. 2002; Parkhill et al. 2001; Song et al. 2004) and their genomes have been available at NCBI and Enteropathogen Resource Integration Center (ERIC; http://www.ericbrc.org/portal/eric/). Sometime in 2010, information from ERIC will migrate to a new bioinformatics resources center PATRIC (PAthoSystems Resources Integration Center; https://patricbrc.vbi.vt.edu). The *Y. pestis* KIM, CO92, and Antiqua strains represent the three classical biotypes (*mediaevalis*, *orientalis*, and *antiqua*, respectively) involved in epidemic outbreaks while Pestoides F and 91001 are isolates of endemic strains from the former USSR and China, respectively, which represent varieties that are less virulent or avirulent in humans (Anisimov et al. 2004; Song et al. 2004). *Y. pseudotuberculosis* PB1/+ and IP32953 are clinical isolates of virulent serotype 1 strains (Burrows and Bacon 1960; Chain et al. 2004).

Some apparent differences in the N-termini of Orfs among the species and/or strains are due to differences in predicted start sites in the annotations. The Enteropathogen Resource Integration Center (ERIC) has been re-evaluating start sites among the *Y. pestis* and *Y. pseudotuberculosis* genome sequences; updated start sites can be found at their website (http://www.ericbrc.org/portal/eric/) and have been used in this analysis.

Experimental analyses

Bacterial strains, storage, and cultivation

Bacterial strains used to conduct experiments in this study are described in Tables 1 and 2. *Y. pestis* KIM6+ (Pgm^+ Lcr^-) is a completely avirulent derivative due

Table 1 Bacterial strains used in this study

	Relevant characteristics[a]	Reference or source
Y. pestis strains		
CO99-3015P	*Δpgm* (Hms$^-$ Ybt$^-$) Lcr$^-$ Pla$^+$	S.W. Bearden
CO99-3015	Pgm$^+$ (Hms$^+$ Ybt$^+$) Lcr$^-$ Pla$^+$	Parkhill et al. (2001), S.W. Bearden
KIM6+	Pgm$^+$ (Hms$^+$ Ybt$^+$) Lcr$^-$ Pla$^+$	Perry et al. (1990), Fetherston et al. (1995)
KIM6-2046.1	Hms$^+$ Ybt$^-$ (*irp2::kan2046.1*) Lcr$^-$ Pla$^+$	Fetherston et al. (1995)
KIM6-2081.1+	Pgm$^+$ (Hms$^+$ Ybt$^+$) Hmu$^-$ (*ΔhmuP'RSTUV*) Has$^-$ (*ΔhasRADE*) Lcr$^-$ Pla$^+$	Rossi et al. (2001)
KIM5(pCD1Ap)+	Ap^r Pgm$^+$ (Hms$^+$ Ybt$^+$) Lcr$^+$ (pCD1Ap; *'yadA::bla*) Pla$^+$	Gong et al. (2001), Rossi et al. (2001)
KIM5-2081.1(pCD1Ap)+	Ap^r Pgm$^+$ (Hms$^+$ Ybt$^+$) Hmu$^-$ (*ΔhmuP'RSTUV*) Has$^-$ (*ΔhasRADE*) Lcr$^+$ (pCD1Ap; *'yadA::bla*) Pla$^+$	Rossi et al. (2001)
Y. pseudotuberculosis strains		
PB1/0	Ybt$^+$ Lcr$^-$	Perry and Brubaker (1983), Perry et al. (1999)
PB1-2046.1/0	Ybt$^-$ (*irp2::kan2046.1*) Lcr$^-$	Perry et al. (1999)

[a] KIM strains with a "+" suffix possess an intact *pgm* locus (i.e., Ybt$^+$ Hms$^+$); KIM strains without this designation are either *pgm* deletions (102-kb of the chromosome) or have mutation(s) within the *pgm* locus. KIM6 strains lack the pCD1 plasmid that encodes a type III secretion system and effector Yops and are Lcr$^-$ while KIM5 strains possess pCD1 or the constructed pCD1Ap and are Lcr$^+$. Ap^r, ampicillin resistant; Hms, biofilm development; Pla, plasminogen activator

to the absence of the low-calcium-response (Lcr) plasmid pCD1 that encodes the Ysc type three secretion system and Yop proteins secreted by the system. *Y. pestis* KIM6-2046.1 is an *irp2::kan2046.1* mutant unable to produce the Ybt siderophore. Strain KIM10+ and the three endogenous KIM plasmids (pMT1, pCD1, and pPCP1) have been sequenced. The KIM10+ strain was derived from KIM6+ by cold-curing of plasmid pPCP1 which encodes the plasminogen activator Pla and the bacteriocin pesticin. (Hu et al. 1998; Lindler et al. 1998; Perry et al. 1990; Perry and Fetherston 1997; Perry et al. 1998, 1999).

Yersinia pestis KIM6-2081.1+ is an Lcr$^-$ *ΔhmuP'RSTUV ΔhasRADE* mutant lacking the ability to use hemin and hemoproteins as an iron source. KIM5(pCD1Ap)+ and KIM5-2081.1(pCD1Ap)+ were constructed by electroporation of pCD1Ap into KIM6+ and KIM6-2081.1+, respectively. In pCD1Ap, an ampicillin resistance cassette is inserted downstream of the frameshift in the pseudogene *yadA* (Gong et al. 2001; Rossi et al. 2001). Plasmid profiles of the reconstituted strains and their phenotype on Congo red agar (Surgalla and Beesley 1969) and magnesium-oxalate plates (Higuchi and Smith 1961) were analyzed (Gong et al. 2001; Rossi et al. 2001). All studies using Pgm$^+$ Lcr$^+$ strains of *Y. pestis* were performed in a CDC-approved BSL3 laboratory following Select Agent regulations and were approved by the University of Kentucky Institutional Biosafety Committee.

Yersinia pestis C099-3015 and a *Δpgm* derivative of this strain (CO99-3015P) were obtained from the CDC and are Lcr$^-$ versions of the sequenced strain CO92. The 102-kb chromosomal *pgm* locus includes the ~28 kb high pathogenicity island (HPI) that encodes the Ybt iron transport system (Buchrieser et al. 1998; Fetherston et al. 1992; Fetherston and Perry 1994; Lesic and Carniel 2004; Lucier and Brubaker 1992).

Yersinia pseudotuberculosis PB1/0 (Ybt$^+$) and PB1-2046.1/0 (Ybt$^-$; *irp2::kan2046.1*) are Lcr$^-$ derivatives of the virulent PB1/+ strain that has been sequenced. The *irp2::kan2046.1* mutation in PB1-2046.1/0 renders it unable to produce the Ybt siderophore (Perry and Brubaker 1983; Perry et al. 1999).

From −80°C glycerol stocks (Beesley et al. 1967), *Y. pestis* strains were grown at 26–30°C on Congo red agar before being transferred to tryptose blood agar base (TBA) slants and grown overnight at 26–30°C. *Y. pseudotuberculosis* strains were inoculated onto TBA slants from −80°C glycerol stocks. Subsequent cultures of both species were grown in Heart Infusion broth (HIB) or deferrated PMH2 (Gong et al. 2001) as indicated.

Table 2 *Y. pestis* and *Y. pseudotuberculosis* proven and putative iron/hemin uptake systems

System name	KIM locus tag number	Functionality[a]				Locus similarities[b]
		F	NF	BF	BNF	
Siderophore-dependent systems						
Yersiniabactin (Ybt)	Y2394-Y2404; Y2584	KIM PB1		All		One pattern
Yersinia non-ribosomal peptide (Ynp)	Y3404-Y3423		CO92[c]	IP	91001	6 Patterns
			KIM[c]	PB1	Ant	
					CO92	
					PF; KIM	
Ysu	Y2633-Y2642		CO92[c]	91001	Ant	2 Patterns
			KIM[c]	CO92	KIM	
				PF; IP		
				PB1		
Aerobactin (Iuc; biosynthesis)	Y3380-Y3384		CO92[c]	IP	91001	2 Patterns
			KIM	PB1	Ant	
			PB1		CO92 PF	
ABC iron/siderophoretransporters						
Yfe	Y1891; Y1894-Y1897	KIM		All		One pattern
Yfu	Y1526-Y1524	KIM		All		One pattern
Yiu	Y2872-Y2875	KIM		All		One pattern
Fit	Y4043-Y4046			All		One pattern
Fiu	Y2837-Y2842			IP	All others	2 Patterns
				PB1		
Fhu and IutA	Y0796-Y0798; Y3385	KIM		All others	PF	2 Patterns
FcuA	Y2556	KIM		All but 91001	91001	2 Patterns
TonB system						
TonB/ExbBD	Y2037; Y3494-Y3495	KIM		All		One pattern
		PB1				
Non-ABC transporters						
Feo	Y3910-Y3912	KIM		All		2 Patterns[d]
Efe	Y2450-Y2452			All others	Ant	2 Patterns
Fet	Y2368-Y2370			All others	PF	2 Patterns
FieF	Y0060			All		One pattern

Table 2 continued

System name	KIM locus tag number	Functionality[a]				Locus similarities[b]
		F	NF	BF	BNF	
Hemin transporters						
Hmu	Y0539-Y0543	KIM		All		One pattern
Has	Y0313-Y0317; Y3919-Y3920; Y3516		KIM	All		One pattern

[a] Funtionality: F, proven functional experimentally; NF, nonfunctionality suggested by experimental evidence; BF, bioinformatics suggests functionality; BNF, bioinformatics suggests nonfunctionality. Strain abbreviations—*Y. pestis*: 91001, 91001; Antiqua, Ant; CO92 and/or CO99-3015, CO92; KIM6+ and/or KIM10+, KIM; Pestoides F, PF; *Y. pseudotuberculosis*: IP32953, IP; PB1/+ and/or PB1/0, PB1

[b] When relatively few amino acid residues changes and/or small deletions or insertions which maintain the ORF occur, they were classified as one pattern

[c] Preliminary experiments in this study suggest nonfunctionality while bioinformatics suggests Ysu is nonfunctional in KIM10+ but may be functional in CO92

[d] One pattern in the seven genomes analyzed for all iron transport systems. However, an eighth genome, *Y. pestis* Angola, is missing *feoC*

Growth in J774A.1 cells

J774A.1 cells were maintained in RPMI1640 media containing 10% fetal calf serum, penicillin, streptomycin, and neomycin (Invitrogen). Two days before infection, 6 well plates were seeded with $\sim 4 \times 10^5$ cells/well.

Yersinia pestis cells were grown at 37°C in deferrated PMH2 through 2 transfers for about 6.5 generations and used to infect J774A.1 cells at an MOI of ~ 10. Prior to infection, the monolayers were washed with PBS, then centrifuged at 1,000 rpm for 3 min with *Y. pestis* cells in RPMI1640 without any additions, and incubated for 1 h at 37°C. The medium was removed and the wells were rinsed once with PBS. External bacteria were killed by incubating the infected tissue culture cells with RPMI1640 containing 7.5 μg/ml of gentamicin for 1 h at 37°C. Duplicate wells were rinsed once with PBS and samples harvested immediately. Infected cell monolayers were maintained in RPMI1640 with 10% FCS and incubated for 1 h with media containing gentamicin prior to harvesting. J774A.1 cells were lysed by incubating with water for 10 min on ice and serial tenfold dilutions of the lysate were plated on TBA.

Chrome azurol S assays

Strains used in the CAS-overlay assay (O-CAS) were passaged twice in PMH2 medium at 37°C and then diluted in fresh PMH2 to an OD_{620} of 0.1 before spotting 25 μl of each dilution onto plates containing PMH2 with 25 μM 2,2′-dipyridyl (DIP) solidified with 0.9% agarose. Cells were grown for 24 h at 37°C before being overlaid with the 10 ml of CAS medium containing 0.9% agarose (Pérez-Miranda et al. 2007) using a modification of the original CAS medium (Schwyn and Neilands 1987). Overlayed plates were incubated at 37°C for 24 h. For *Y. pseudotuberculosis* PB1 strains, the phosphate content of PMH2 was reduced tenfold to 250 μM. The original liquid CAS assay (Schwyn and Neilands 1987) was also used in some trials.

Virulence testing

Cells used for intranasal infections were grown overnight at 37°C in HIB with 2.5 mM $CaCl_2$, diluted in fresh media to an OD_{620} of 0.1 and harvested during

mid-exponential phase (OD_{620} of ~0.3–0.5). Harvested cells were 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; Harlan) mice were infected intranasally with serially diluted bacterial suspensions ranging from 5×10^3 to 5×10^6 cfu/ml. Twenty microliters of the bacterial suspension was administered to the nares of mice sedated with 100 mg of ketamine and 10 mg of xylazine/Kg of body weight. The actual administered dose was 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 LD_{50} values were calculated according to the method of Reed and Muench (Reed and Muench 1938). All animal care and experimental procedures were conducted in accordance with the *Animal Welfare Act*, *Guide for the Care and Use of Laboratory Animals, PHS Policy and the U.S. Government Principals for the Utilization of and Care for Vertebrate Animals in Teaching, Research, and Training* and approved by the University of Kentucky Institutional Animal Care and Use Committee. The University of Kentucky Animal Care Program first achieved accreditation by 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

Detailed analysis of the KIM genome has identified 16 putative iron transport systems. Of 9 systems that have been experimentally examined, 7 are functional. (Bearden et al. 1997; Bearden and Perry 1999; Fetherston et al. 1999; Forman et al. 2007; Gong et al. 2001; Hornung et al. 1996; Kirillina et al. 2006; Perry 2004; Perry and Fetherston 2004; Rossi et al. 2001). The 16 systems can be divided into: (1) siderophore-dependent systems; (2) ABC iron transporters; (3) non-ABC iron transporters; and (4) hemin transporters (Table 2). These systems in *Y. pestis* KIM were compared to their homologues in *Y. pestis* strains CO92, Antiqua, Pestoides F, and 91001 as well as *Y. pseudotuberculosis* strains PB1/+ and IP32953.

Siderophore-dependent systems

Siderophores are small molecular weight compounds that are enzymatically synthesized and secreted into the environment where they bind ferric iron. The Fe-siderophore complex is generally transported into the bacterial cell where the iron is removed and used metabolically.

The Yersiniabactin system

The Ybt system (Fig. 1) is essential for virulence of *Y. pestis* from peripheral infection sites (subcutaneous, intradermal, intraperitoneal) in mice. Biosynthetic, transport, and regulation mechanisms have been extensively studied in *Y. pestis* and *Y. enterocolitica* and recently reviewed (Perry 2004; Perry and Fetherston 2004). This system is wide-spread among bacterial pathogens probably because it is carried on a pathogenicity island. In *Y. pestis,* this pathogenicity island (HPI) lies within the larger *pgm* locus, a 102-kb chromosomal region that undergoes spontaneous deletion due to recombination between two IS*100* elements that bound the locus (Brubaker 1969; Buchrieser et al. 1998; Fetherston et al. 1992; Fetherston and Perry 1994; Hare and McDonough 1999; Lesic and Carniel 2004; Lucier and Brubaker 1992). Although the system has not been extensively studied in *Y. pseudotuberculosis*, the Ybt system is functional and a mutation in a biosynthetic gene causes an ~1,000-fold or greater loss of virulence in mice infected subcutaneously or intravenously. This is in contrast to *Y. pestis* KIM where a *Δpgm* mutant is fully virulent by an intravenous infection route in mice (Carniel et al. 1992; Perry et al. 1999; Une and Brubaker 1984). In the 7 genomes analyzed, no significant differences were found in the *ybt* locus (Fig. 1a).

The Yersinia non-ribosomal peptide (Ynp) locus

The *ynp* locus has not been tested for function. A recent microarray analysis of a Microtus strain indicated Fur-dependent iron regulation of the homologous locus supporting a role in siderophore biosynthesis and transport (Gao et al. 2008). There are intriguing differences among the genomes analyzed. *Y. pestis* KIM10+, CO92, and Antiqua (all three epidemic biotypes) have an IS*100* element and

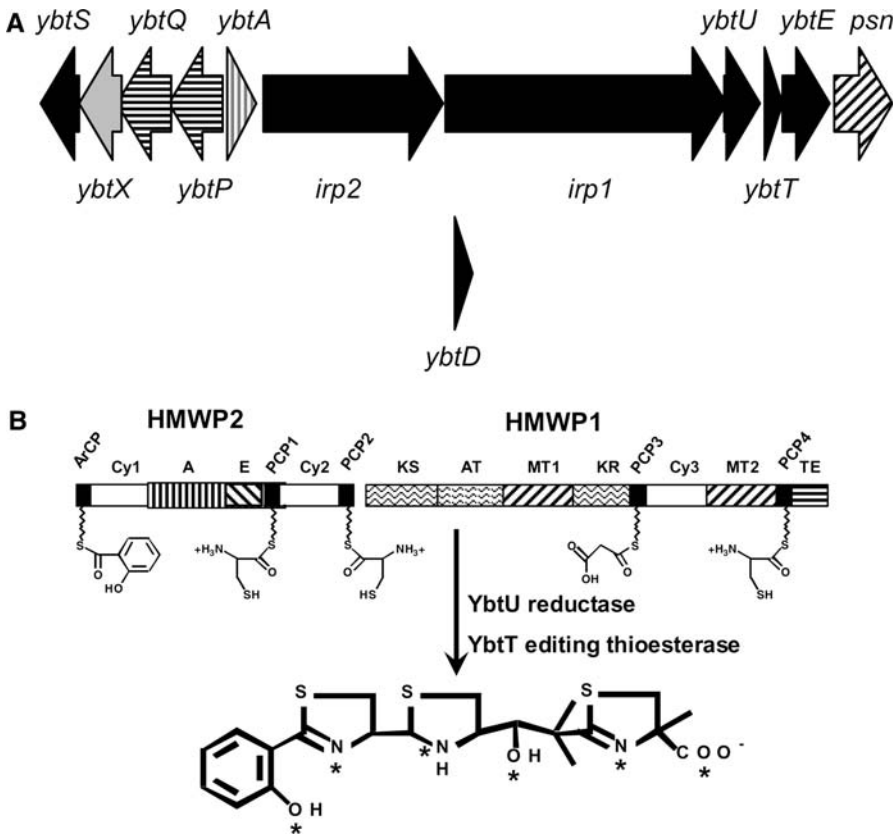


Fig. 1 The Yersiniabactin (Ybt) locus. From *left* to *right* the gene designations in *panel A* correspond to *y2394-y2404* and *y2584* (*second line*) in *Y. pestis* KIM. The *ybtD* gene is shown on a separate line since it is encoded at a separate locus from the other *ybt* genes. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding biosynthetic enzymes (*black*), inner membrane (IM) fused function permeases/ATPases (*horizontal lines*), an outer membrane (OM) receptor (*diagonal lines*), a transcriptional regulator (*vertical lines*), and a protein of unproven function, YbtX (*grey*). *Panel B* shows the assembly line production of Ybt. The HMWP1 and HMWP2 attachment sites for salicylate, malonate, and the three cysteine residues that are precursors for the thiazolidine and two thiazoline rings are indicated. Reactions prior to this stage involving YbtS (salicylate biosynthesis), YbtE (adenylates salicylate), and YbtD (phosphopantetheinyl transfer to HMWP1 and HMWP2) are not shown. Enzymatic domains: ArCP, aryl carrier protein; Cy, condensation/cyclization; A, adenylation; E, epimerization; PCP, peptidyl carrier protein; KS, ketoacyl synthase; AT, acyltransferase; MT, methyltransferase; KR, *β*-ketoreductase; TE, thioesterase. The proven ferric ion coordination sites of the completed Ybt siderophore (Miller et al. 2006) are indicated by asterisks. Panel B is reproduced from Perry and Fetherston (Perry and Fetherston 2004) with the permission of Horizon Bioscience

a frameshift disrupting the second ORF. KIM10+ has a second frameshift mutation in this same ORF (Fig. 2), while in CO92, a likely recombination event between IS*100* elements has resulted in the *ynp* locus being encoded in two separate regions of the chromosome (indicated by the 2 lines in Fig. 2). In contrast, the *ynp* loci of *Y. pestis* 91001 and *Y. pseudotuberculosis* IP32953 and PB1 do not have an IS*100* insertion. However, *Y. pestis* 91001 has the same frameshift mutation as CO92 and Antiqua in the second ORF (Fig. 2). Finally, *Y. pestis* Pestoides F lacks this locus entirely. Thus, it appears that this locus may produce a siderophore-like molecule in *Y. pseudotuberculosis* but not in any of the biotypes of *Y. pestis*.

Using similarities to characterized proteins in the literature/database (Ansari et al. 2004; Crosa and Walsh 2002; Walsh and Marshall 2004), we can predict the assembly and the structure of the *Y. pseudotuberculosis* putative natural product via the mixed nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) mechanism (Fig. 3). Since

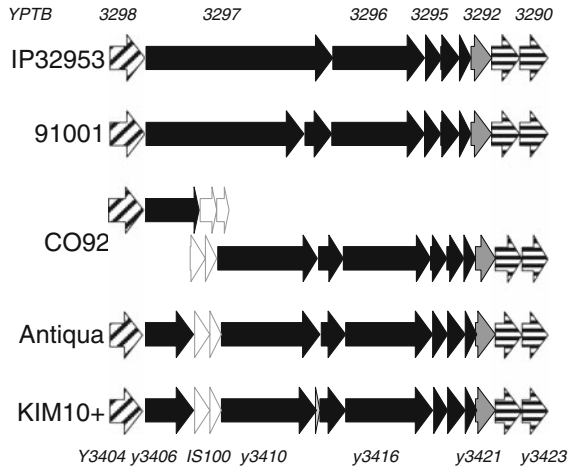


Fig. 2 The *ynp* locus appears to be intact only in *Y. pseudotuberculosis* strains IP32953 and PB1/+. In all *Y. pestis* strains, except Pestoides F, the locus is disrupted by an IS*100* and/or a frameshift mutation. The *ynp* locus is not encoded in the Pestoides F genome. In CO92 a likely recombination event between IS*100* elements has resulted in the *ynp* locus being encoded in two separate regions of the chromosome (indicated by the *2 lines*). *Arrows* show the direction of transcription and *arrow fills* identify genes encoding biosynthetic enzymes (*black*), IM fused function permeases/ATPases (*horizontal lines*), OM receptors (*diagonal lines*), transposase ORFs (*open*), and proteins of unknown function (*grey*). Small ORFs due to frameshift mutations are assigned the function of the intact ORF. *Y. pestis* strains shown are 91001, CO92, Antiqua, and KIM10+. Selected locus tags from *Y. pseudotuberculosis* IP32953 (YPTB numbers) and *Y. pestis* KIM10+ (*y* numbers) are shown

this locus does not encode a phosphopantetheinyl (P-pant) transferase for initiating assembly on the NRP/PKS complex, it would need to use one of the two P-pant transferases encoded within the *Yersinia* genomes—YbtD which initiates the Ybt system or AcpP for fatty acid synthesis (Bobrov et al. 2002). The predicted structure in Fig. 3 may not be accurate but it will provide a guide for physical purification and identification of the putative natural product(s). Similarities among the Ybt and Ynp Orfs are numerous. YBTB3297 and YBTB3296 have similar enzymatic domains to those in HMWP2 and HMWP1. This is reflected in the predicted structure of the putative Ynp product (compare Figs. 1b, 3) which has three thiazoline rings. Both loci encode an outer membrane (OM) receptor (Psn and Y3404 in KIM10+; Figs. 1, 2), two unusual fused function permease/ATPase inner membrane (IM) proteins (YbtP/YbtQ and Y3422/Y3423) and an IM protein of unproven function (YbtX and Y3421). These proteins encoded by the *ynp* locus exhibit significant similarities to their Ybt counterparts.

Yersinia siderophore uptake (Ysu) locus

Although no experimental analysis has been performed on this locus in any isolate, microarray analysis in a Microtus strain suggests Fur-dependent iron regulation of the homologous locus (Gao et al. 2008). The genomes fall into two classes (Fig. 4). A frameshift mutation has occurred in the first ORF in *Y. pestis* KIM and Antiqua. The locus appears intact in *Y. pestis* CO92, 91001, Pestoides F as well as *Y. pseudotuberculosis* IP32953 and PB1/+. Amino acid residues in all other ORFs are identical in all 7 organisms.

Similarities to other characterized enzymes in the literature/database can be used to construct putative biosynthetic pathways and structures of the hypothetical product (Challis 2005; Walsh and Marshall 2004) in the *Yersinia* in which the locus appears intact (Fig. 4). Two alternative biosynthetic pathways can be postulated with four alternative hydroxamate siderophore structures (Fig. 5). While the structures may not be accurate, they could be used as a guide for purification and identification of the putative product(s).

CAS-reactive products secreted by Y. pestis

Our computational analysis suggests that *Y. pestis* CO92 could synthesize a product from the *ysu* locus while *Y. pseudotuberculosis* strains may make a Ynp molecule in addition to a Ysu product. Neither of these putative siderophores are made in other epidemic biotypes of *Y. pestis.* To detect potential siderophores made by *Y. pestis* KIM, CO92, and *Y. pseudotuberculosis* strains, we used the O-CAS (overlaid chrome azural S) assay. In this assay, cells acclimated to iron-deficient conditions are spotted onto deferrated PMH2 plates, allowed to grow, and then overlaid with CAS medium to detect removal of iron from the chromophore, presumably by a siderophore. Initial results showed that KIM6+ and CO99-3015 produced a yellow halo that diffused away from the area of bacterial growth (Fig. 6). However, the KIM6-2046.1 *irp2* mutant (Ybt^{-}) produced no halo indicating that *Y. pestis* KIM only

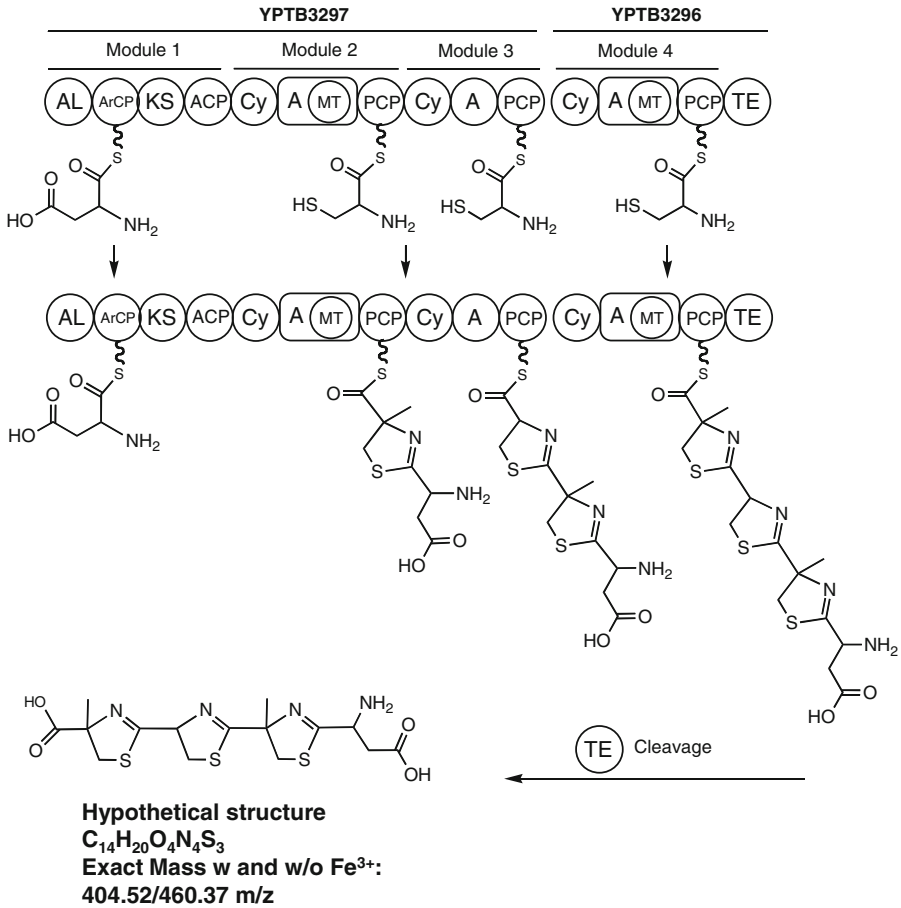


Fig. 3 Predicted assembly and structure of a hypothetical siderophore produced by the *Y. pseudotuberculosis ynp* locus. The predicted siderophore belongs to a third class of siderophores with oxazoline, thiazoline, and/or thiazolidine rings. The putative Ynp siderophore has three thiazoline rings and thus has some structural similarities to the Ybt siderophore. Abbreviations for enzymatic domains are the same as in Fig. 1

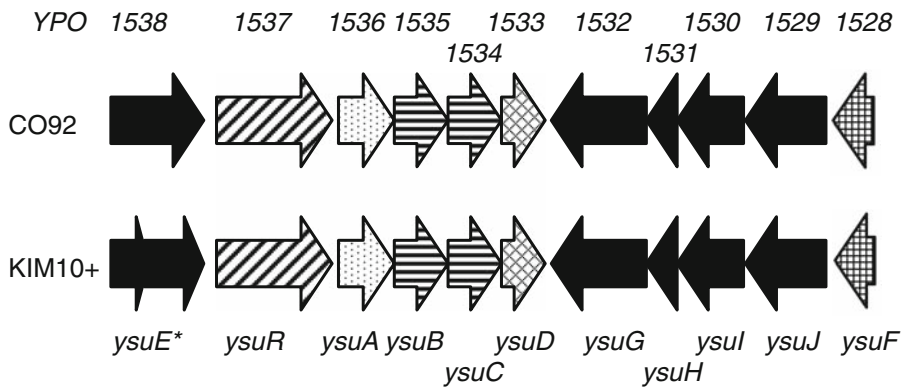


Fig. 4 The *ysu* locus appears to be intact in *Y. pseudotuberculosis* and *Y. pestis* strains except for KIM and Antiqua which have a frameshift mutation in the first ORF. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding biosynthetic enzymes (*black*), IM permeases (*horizontal lines*), an ATP-binding protein (ATPase; *diamonds*), a periplasmic binding protein (PBPs) (*dots*), an OM receptor (*diagonal lines*), and a ferric reductase (*squares*). Selected locus tags from *Y. pestis* CO92 (*YPO* numbers) and *Y. pestis* KIM10+ (*ysu* designations) are shown. *ysuE** is used to designate two Orfs resulting from a frameshift in KIM10+

produces the Ybt siderophore. Similarly, *Y. pestis* CO99-3015P (a *Δpgm* mutant which cannot produce Ybt) failed to make a compound capable of removing iron from CAS. Note that the diffusion zone due to Ybt production is much larger in CO99-3015 than in KIM6+ suggesting a higher level of siderophore production in CO92 (Fig. 6). Since this might have been caused by differences in the promoters for *ybt* genes or in Fur, we examined both these characteristics in CO92 and KIM6+ and found that all the promoter regions of *ybt* genes as well as the predicted amino acid sequence of Fur were identical.

Unlike *Y. pestis,* results for *Y. pseudotuberculosis* PB1/0 were inconsistent even though this strain is known to produce the Ybt siderophore (Carniel et al. 1992; Perry et al. 1999; data not shown). Since phosphate can interfere with the CAS reaction, we tested solidified PMH2 medium containing 250 and 2.5 mM phosphate (the normal concentration). The lower level of phosphate did not inhibit the growth of *Y. pseudotuberculosis* or *Y. pestis* (data not shown). The *Y. pestis* strains yielded similar results—*irp2* mutants and *Δpgm* strains failed to indicate production of any other siderophore. With the lower phosphate concentration, *Y. pseudotuberculosis* PB1/0 occasionally exhibited a substantial diffusion zone presumably due to siderophore production. *Y. pseudotuberculosis* PB1-2046.1/0 (an *irp2* mutant) also occasionally had a zone suggesting that a non-Ybt siderophore was being produced (data not shown). However, in other trials, neither strain showed siderophore production. The use of a liquid CAS assay also gave inconsistent results for *Y. pseudotuberculosis,* but not *Y. pestis*. Thus we could not determine whether *Y. pseudotuberculosis* PB1/0 makes any siderophore in addition to Ybt.

The aerobactin locus

The aerobactin biosynthetic (*iucA-D*) and OM receptor (*iutA*) locus appears intact in the *Y. pseudotuberculosis* genomes. The Fhu ABC transporter for aerobactin and some other siderophores (e.g., ferrichrome) is discussed in the ABC transporter section below since it is encoded in a separate locus.

A frameshift in *iucA* has occurred in all *Y. pestis* strains examined. In some genomes (e.g., CO92 and 91001), the second frameshifted ORF is present but not annotated (Fig. 7). The remaining aerobactin biosynthetic genes appear intact.

Fig. 5 Two potential biosynthetic pathways (*Panels A* and *B*)▶ for the production of a putative hydroxamate siderophore by an intact *ysu* locus. Each of the two potential pathways (*panels A* and *B*) could yield two different structures

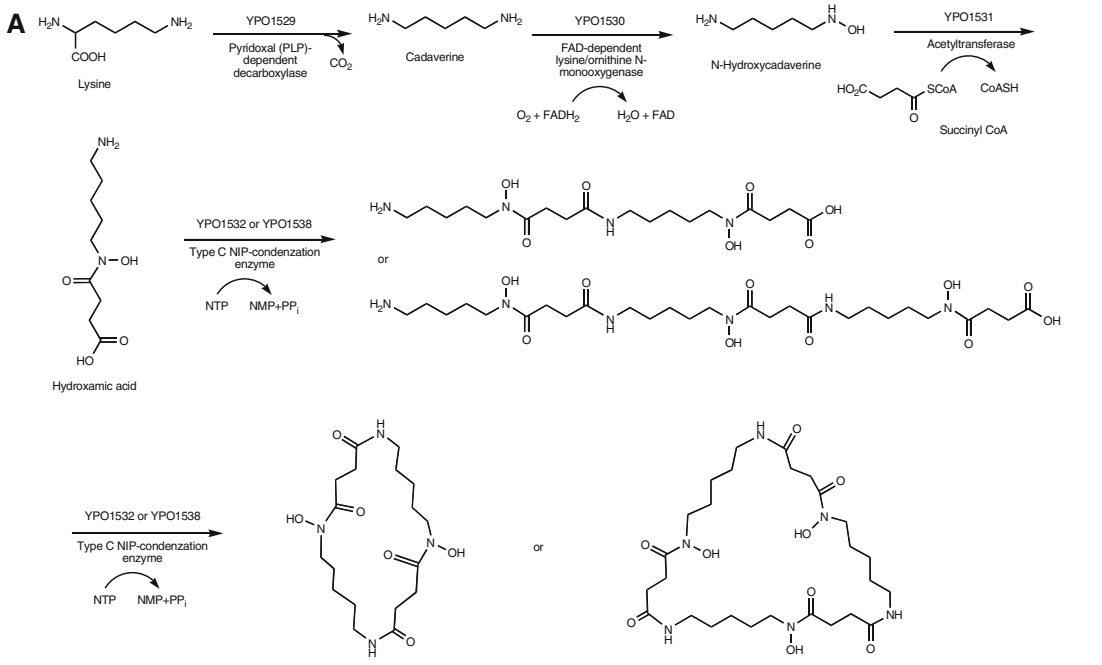
The *Y. pestis iucA-D* operon was not able to complement an *iucB* mutation in *E. coli* suggesting that one or more of the *iucB-D* genes encode defective enzymes. However, expression of a functional aerobactin biosynthesis locus from *E. coli* or *Shigella* allows synthesis and use of aerobactin as a siderophore in *Y. pestis* KIM (Forman et al. 2007). In vitro transcription/translation analysis indicates that the downstream *iucBCD* gene products of the KIM aerobactin locus are not produced, likely due to the *iucA* frameshift. However, the IutA (OM receptor) protein is produced suggesting that its expression is not linked to either transcription or translation of the upstream genes. The *iucA* promoter is transcriptionally active and repressed by iron in a Fur-dependent mechanism (Forman et al. 2007).

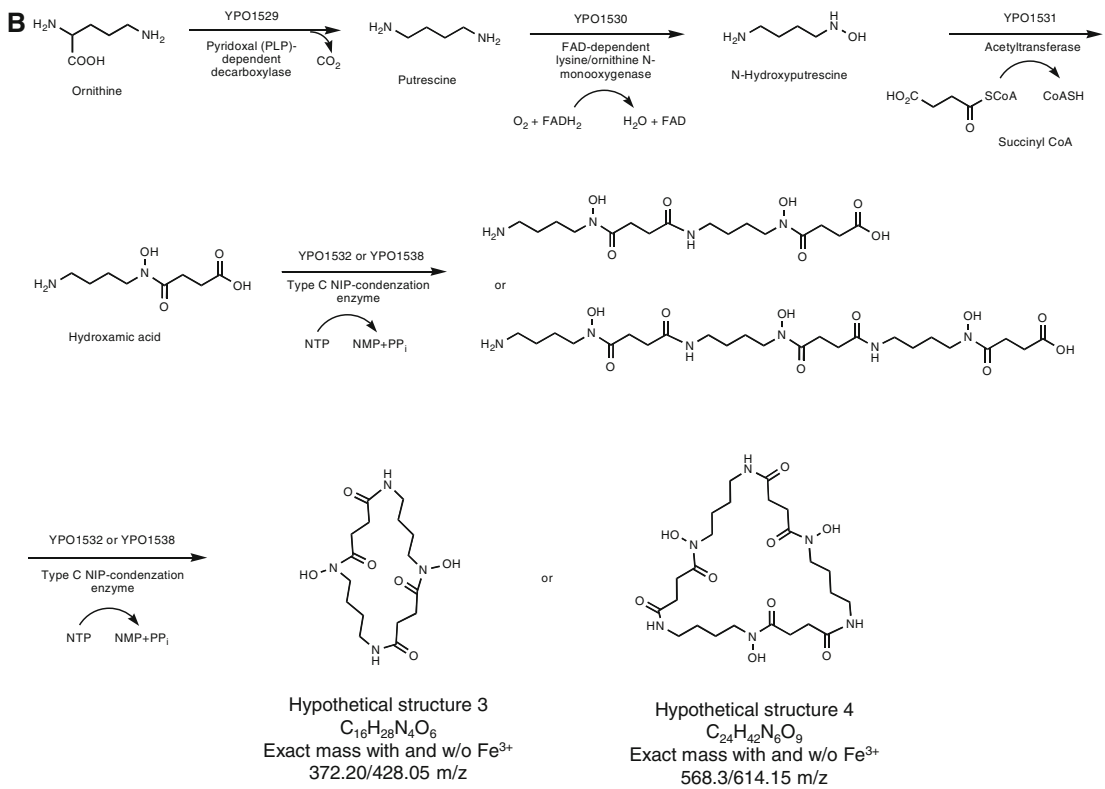
Strain KIM does not produce detectable aerobactin by a bioassay. Correction of the frameshift mutation in *iucA* resulted in the production of IucB,C, and D proteins in an in vitro assay. However, these changes do not lead to aerobactin production/secretion in either *E. coli* or *Y. pestis* KIM. Although the *Y. pestis* and *Y. pseudotuberculosis* IucB, C, and D amino acid sequences are identical, there are several amino acid differences in the *Yersinia* sequences compared to known functional Iuc enzymes from *E. coli* and *Shigella.*

Although the aerobactin biosynthetic genes of *Y. pseudotuberculosis* have no obvious defects, bioassays do not detect synthesis and secretion of aerobactin. While there are numerous possible explanations for this result, the most likely reason is that amino acid changes in *Y. pestis* and *Y. pseudotuberculosis* IucA-D enzymes, compared to functional Iuc enzymes from *E. coli* and *Shigella*, make one or more of these proteins enzymatically inactive (Forman et al. 2007).

ABC transporters for iron/siderophores

ABC transporters for substrate accumulation are generally composed of a solute-binding protein (or


A
Lysine
YPO1529
Pyridoxal (PLP)-dependent decarboxylase
CO2
Cadaverine
YPO1530
FAD-dependent lysine/ornithine N-monooxygenase
O2 + FADH2
H2O + FAD
N-Hydroxycadaverine
YPO1531
Acetyltransferase
CoASH
Succinyl CoA
Hydroxamic acid
YPO1532 or YPO1538
Type C NIP-condenzation enzyme
NTP
NMP+PPi
or
YPO1532 or YPO1538
Type C NIP-condenzation enzyme
NTP
NMP+PPi
or
Hypothetical structure 1
C16H32N4O6
Exact Mass with and w/o Fe3+:
400.23/456.08 m/z
Hypothetical structure 2
(Desferrioxamine E)
C27H48N6O0
Exact Mass with and w/o Fe3+:
600.35/656.20 m/z


B
Ornithine
YPO1529
Pyridoxal (PLP)-dependent decarboxylase
CO2
Putrescine
YPO1530
FAD-dependent lysine/ornithine N-monooxygenase
O2 + FADH2
H2O + FAD
N-Hydroxyputrescine
YPO1531
Acetyltransferase
CoASH
Succinyl CoA
Hydroxamic acid
YPO1532 or YPO1538
Type C NIP-condenzation enzyme
NTP
NMP+PPi
or
YPO1532 or YPO1538
Type C NIP-condenzation enzyme
NTP
NMP+PPi
or
Hypothetical structure 3
C16H28N4O6
Exact mass with and w/o Fe3+
372.20/428.05 m/z
Hypothetical structure 4
C24H42N6O9
Exact mass with and w/o Fe3+
568.3/614.15 m/z

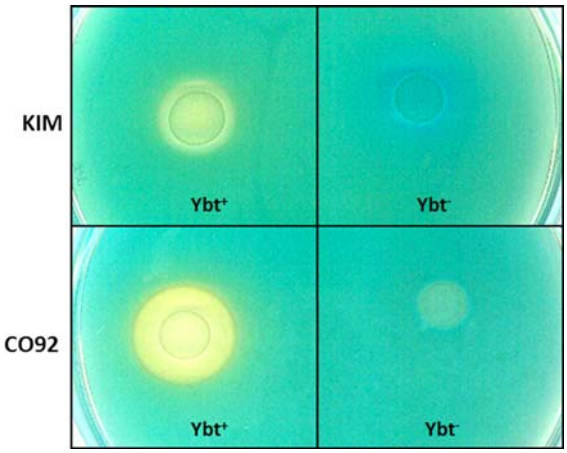


Fig. 6 O-CAS assay for siderophore detection. Strains of KIM and CO92 were grown in deferrated PMH2 at 37°C, spotted onto PMH2—25 μM DIP. Cells were grown for 24 h at 37°C before being overlaid with the 10 ml of CAS medium containing 0.9% agarose (Pérez-Miranda et al. 2007) using a modification of the original CAS medium (Schwyn and Neilands 1987). Overlayed plates were incubated at 37°C for 24 h. Zones around cell growth indicate secretion of a compound capable of removing iron from the chromophore. Ybt^+ and Ybt^- strains of *Y. pestis* KIM and CO92 are shown

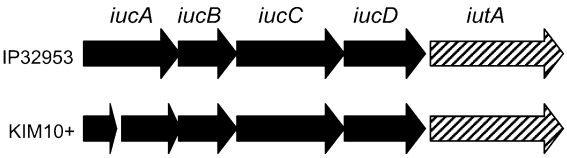


Fig. 7 The *iucABCD-iutA* locus. The first gene (*iucA*) has undergone a frameshift mutation in all *Y. pestis* strains. Although the locus in *Y. pseudotuberculosis* strains IP32953 and PB1/+ appears intact, PB1/0 does not synthesize aerobactin (see text). PB1/0 is a derivative of PB1/+ that has been cured of the virulence plasmid. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding biosynthetic enzymes (*black*) and the IutA OM receptor (*diagonal lines*)

periplasmic-binding protein [PBP]) in the periplasm along with an IM permease and an IM-associated ATP-binding protein or ATPase. Some ABC systems have 2 essential permeases while others have 2 different ATPases.

Of the 6 ABC transporters with similarities to inorganic iron or siderophore transporters (Figs. 8, 9), 4 are proven iron transporters (Fhu, Yfe, Yfu, and Yiu) with Yfe also transporting manganese. YfeE, an IM protein, is not essential but enhances the function of the Yfe ABC transporter. Under aerobic conditions, there is an efficacy hierarchy in tested

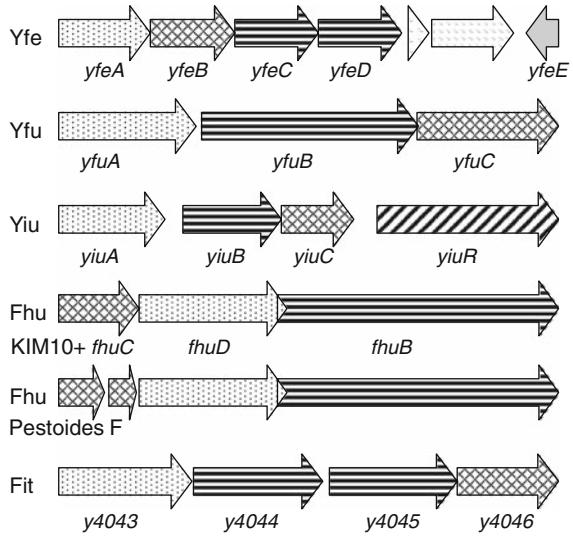


Fig. 8 ABC inorganic iron/siderophore transporters. Yfe, Yfu, and Yiu are experimentally proven iron transporters while Fhu transports aerobactin and ferrichrome. The Fit system has not been tested. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding IM permeases (*horizontal lines*), ATPases (*diamonds*), PBPs (*dots*), an OM receptor (*diagonal lines*), an IM protein of unknown function (YfeE; *grey*), and intervening Orfs (Y1892 and Y1893; grey dashes). Except for *fhuCDB*, the gene order is A, B, C, (D, E) with A always encoding a PBP. The gene encoding the ATP-binding protein is generally the final gene of the operon with permeases encoded in the second (and third of four) gene(s). The *yfe* locus is an exception with the ATPase encoded by the second gene with the third and fourth each encoding essential permease components; it also has a second *yfeE* operon. The *yiu* locus also encodes an OM receptor (YiuR)

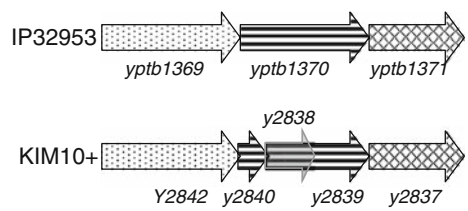


Fig. 9 The *fiu* locus has two variations with only the *Y. pseudotuberculosis* IP32953 and PB1 loci likely to be functional. The *Y. pestis* strains appear to have a frameshift in the second gene, encoding a permease. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding IM permeases (*horizontal lines*), ATPases (*diamonds*), and PBPs (*dots*). The *shadow arrow* in KIM10+ indicates a small ORF annotated within the primary gene

transporters of *Y. pestis* KIM6+ for inorganic iron—Ybt > Yfe > Yfu > Yiu. The YiuR OM receptor shows similarity to *V. cholerae* IrgA which is involved in the use of enterobactin. Neither the Yfu

nor the Yiu systems have significant in vivo roles in a mouse model of bubonic plague. FhuCDB has been proven to transport aerobactin and ferrichrome. While transcription of all 6 systems appears to be repressed by iron through Fur, the *yfeA-D* operon is also repressed by manganese via Fur. In contrast, *yfeE* has an apparent Fur-binding site but is not regulated by Fur or iron (Bearden et al. 1998; Bearden and Perry 1999; Forman et al. 2007; Gao et al. 2008; Gong et al. 2001; Kirillina et al. 2006; Mey et al. 2002; Perry et al. 2003a). In *Shigella flexneri,* the homologous *sitA-D* operon is also repressed by manganese and iron, partially through Fur (Runyen-Janecky et al. 2003). However, in other bacteria manganese-repression of manganese transporters occurs through MntR, a DtxR-family transcriptional regulator (Papp-Wallace and Maguire 2006). MntR is not encoded by the *Y. pestis* KIM genome (Deng et al. 2002).

The Yfe, Yfu, Yiu and Fit systems are identical in all *Y. pestis* strains with 1–2 amino acid residue changes in 1–3 ORFs in both *Y. pseudotuberculosis* genomes. The Fhu system is identical in all but *Y. pestis* Pestoides F where a frameshift in the first ORF (*fhuC*) of the operon has occurred (Fig. 8). Both *Y. pseudotuberculosis* genomes appear to have an intact *fiu* locus while the *Y. pestis* strains have a frameshift disrupting the second ORF in the locus. In all but KIM, the first part of the disrupted ORF is no longer apparent (Fig. 9).

TonB system and isolated transport components

TonB and ExbBD

A class of OM receptors (called TonB-dependent) require the TonB/ExbBD system to translocate their substrates through the OM. In *Y. pestis* KIM, the Ybt and Hmu systems have TonB-dependent receptors and have been proven to be TonB-dependent while the Yfe system is independent of TonB and HasB (a second TonB-like protein). Transport of the siderophores aerobactin and ferrichrome are likely TonB-dependent since they use putative TonB-dependent receptors (Forman et al. 2007; Perry et al. 2003b). The TonB amino acid sequence is identical in all 5 *Y. pestis* strains while *Y. pseudotuberculosis* IP32953 and PB1/+ have a 4 residue deletion (ΔVPVP, residues 102–105). However, this deletion does not affect TonB function since the Ybt transport system in *Y. pseudotuberculosis* PB1 is functional (Perry et al. 1999). ExbB is identical in all but IP32953, PB1/+, and Pestoides F which have the same residue change (I271 V). ExbD is identical in all *Y. pestis* strains with a T88A change in IP32953. Again, these changes are likely inconsequential since the TonB-dependent Ybt system is functional in *Y. pseudotuberculosis* (Perry et al. 1999).

Isolated receptor and ABC transport components

Most ABC transporter components are encoded within the same locus, frequently with OM receptors. However, FcuA (Y2556 in KIM), the TonB-dependent receptor for ferrichrome, is encoded separate from the *fhuCDB* locus. Compared to most *Y. pestis* strains (except 91001), the *Y. pseudotuberculosis* FcuA has 7 residue changes and a 5 amino acid residue insertion. The *Y. pestis* 91001 *fcuA* gene is disrupted by an IS*100* insertion and subsequent rearrangement. Another isolated putative TonB-dependent OM receptor (Y0850) has one residue change in CO92 and a frameshift in Antiqua but an identical sequence in the remaining *Yersinia* genomes. Y3342 and Y3343 are adjacent genes encoding a PBP and TonB-dependent OM receptor, respectively. They are identical in all 5 *Y. pestis* genomes examined with *Y. pseudotuberculosis* IP32953 and PB1/+ exhibiting one residue change in the homologue of Y3342. For the Y3343 homologue, *Y. pseudotuberculosis* IP32953 has 2 residue changes while PB1 has 3 amino acids different from *Y. pestis* KIM. The two *Y. pseudotuberculosis* strains differ from each other at 3 amino acid residues. Finally, a putative PBP (Y3477 in *Y. pestis* KIM) is identical in all 5 *Y. pestis* genomes examined and *Y. pseudotuberculosis* IP32953. However, the PB1/+ equivalent (YPTS_3497) has 7 residues changes compared to the other 6 genome sequences. These are the only two instances (Y3343 and Y3477) of differences between the two serotype I *Y. pseudotuberculosis* genomes that we found. They might simply represent cumulative mutations from decades of cultivation in the laboratory.

Non-ABC iron transporters

There are four systems that are not typical ABC transporters with one of these being a putative

efflux system (FieF; Y0060). The Feo ferrous transporter has been studied in a number of organisms including *Y. pestis* (Cartron et al. 2006; Hantke 2004; Perry et al. 2007). There are two ILT superfamily members (Efe and Fet) probably of the OFeT family exemplified by the yeast FTR—*E. coli* Efe systems and the *E. coli* FetM/FetP-M*agneto-spirillum* MV-1 systems (Cao et al. 2007; Dubbels et al. 2004; Große et al. 2006; Ollinger et al. 2006; Koch et al. 2008). Neither system has been tested in *Yersinia*.

FeoABC

FeoB is an IM GTP-binding protein while FeoA is an essential small protein. In *Y. pestis* KIM this system transports iron under microaerophilic conditions and has somewhat overlapping or redundant functions with the Yfe system. FeoC is not required for Feo to function in *Y. pestis* KIM (Perry et al. 2007). This system has one genetic pattern and is identical in 6 of the 7 reference organisms—*Y. pestis* Pestoides F has 2 residues changes in FeoB (Fig. 10a). Although the *Y. pestis* Angola genome was not routinely analyzed, we noticed that *feoC* is missing (not shown in Fig. 10a).

EfeUOB (Y2450–Y2452)

This system shows similarity to the *E. coli* Efe system (Große et al. 2006). In *E. coli,* EfeU functions as an IM permease while EfeO and EfeB are periplasmic proteins with EfeB possessing a peroxidase/oxidoreductase domain (Cao et al. 2007; Große et al. 2006). The 3 ORFs are identical in 4 *Y. pestis* strains (Fig. 10b)—Antiqua has a frameshift in the homologue to Y2450 while *Y. pseudotuberculosis* IP32953 and PB1 have one amino acid change in 2 ORFs and 2 changes and a 6-bp (2-amino acid) deletion in the third ORF compared to *Y. pestis* KIM10+.

FetMP (Y2370 and Y2368)

Recently, FetM and FetP (*EcolF_1003470* and *EcolF_1003469* in *E. coli* F11) were shown to function in the uptake of ferrous iron in *E. coli* (Koch et al. 2008). Y2370 and Y2368 are the corresponding homologues in *Y. pestis* KIM. Y2368, a predicted periplasmic protein, is identical in all 7 genomes while Y2370, a predicted permease, is identical in 6 of the strains with a 10-bp deletion causing a frameshift in *Y. pestis* Pestoides F. *Y. pestis* KIM has an annotated short 67-amino-acid ORF (Y2369) between the other two ORFs that would be transcribed in the opposite direction (Fig. 10c). This ORF is present but not annotated in the other 5 genomes; its function or even translation into a protein is undetermined.

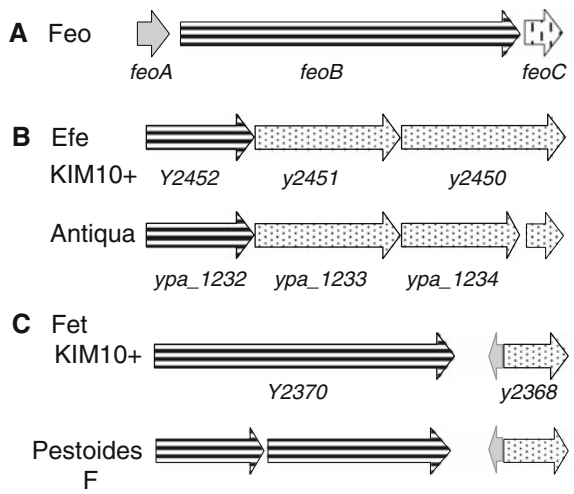


Fig. 10 Feo and ILT superfamily transporters Efe and Fet. **a** FeoB may be a permease—although this putative function is disputed. The *Y. pestis* Angola locus which lacks *feoC* is not shown. **b** We propose designating the KIM Y2452-Y2460 locus tags as EfeNOB$_{Yp}$ due to their high similarity to the *E. coli* EfeNOB system. In *Y. pestis* Antiqua, there is a frameshift in the Orf corresponding to Y2450. **c** While Y2368 and Y2370 also belong to the ILT superfamily it shows a higher similarity to ferrous transporters in *E. coli* (FetM/FetP) and a magnetotactic bacterium. *Arrows* show the direction of transcription and *arrow fills* identify genes encoding IM proteins (*horizontal lines*) and periplasmic proteins (*dots*). FeoA (*grey*) is essential but of unproven function while FeoC (*vertical dashes*) is not essential in the *Y. pestis* KIM strain

FieF (Y0060)

Recently, overexpression of *E. coli fieF* (formerly *yiiP*) was shown to reduce accumulation of Fe and cause active efflux of Zn. From these and other data it was suggested that *E. coli* FieF is an efflux system for Fe and Zn (Grass et al. 2005). In the seven *Yersinia* genomes analyzed, the FieF Orfs are identical. No experimental studies have been performed on this locus in the *Yersinia.*

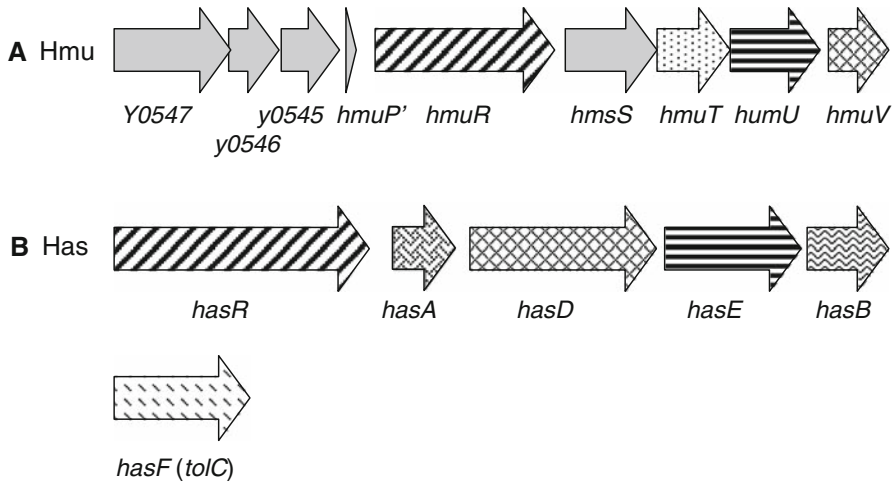


Fig. 11 The *hmu* and *has* loci are identical or extremely similar in all seven *Yersinia* genomes analyzed. *Arrows* show the direction of transcription and *arrow fills* in panel A identify genes encoding IM permeases (*horizontal lines*), ATPases (*diamonds*), PBPs (*dots*), OM receptors (*diagonal lines*), and proteins of unproven function in *Y. pestis* and *Y. pseudotuberculosis* (*grey*). The Has system (*panel B*) is composed of an OM receptor (HasR), a TonB-like protein (HasB), the secreted HasA hemophore and Type I secretion components for HasA that include the HasE permease, the HasD ATPase, and the chunnel protein annotated as TolC or HasF. The *hasRADEB* locus is separate from *hasF*

Hemin transporters

Hmu ABC transporter

The *Yersinia* Hmu system is typical of the many hemin transporters that have been studied in Gram-negative bacteria. The proteins encoded by *hmuR-STUV* are identical in all 5 *Y. pestis* genomes (Fig. 11a) with HmuR from both *Y. pseudotuberculosis* genomes showing 4 amino acid residue changes and 2 changes in HmuT. HmuTUV comprise the ABC transporter with HmuR serving as the OM receptor. A small 41 amino acid ORF (HmuP') appears to serve no function, so differences in this ORF among the *Yersinia* were not analyzed. In *Yersinia enterocolitica*, the essentially identical system has been extensively characterized and designated HemRSTUV (Perkins-Balding et al. 2004; Perry and Fetherston 2004). Homologues of HmuS appear to serve as a hemin-binding protein to prevent toxicity in other enteric bacteria. In addition, three upstream Orfs have been implicated in iron release from hemin in these enterics. The *Yersinia* have all three of these upstream ORFs (Y0545-Y0547 in KIM; Fig. 11a). (Di Lorenzo et al. 2004; Payne and Mey 2004; Perkins-Balding et al. 2004; Wyckoff et al. 2004).

Although no studies on this specific system have been performed in *Y. pseudotuberculosis*, strain PB1 can utilize hemin (Perry and Fetherston 2004). While the Hmu system is required for use of hemin and a variety of hemoproteins in *Y. pestis* KIM, an *hmu* mutation did not affect virulence in a mouse model of bubonic plague (Hornung et al. 1996; Rossi et al. 2001; Thompson et al. 1999). We have now tested an *hmu has* double mutant (see Has hemophore system below) in an intranasal mouse model of pneumonic plague. In this model, the LD_{50} of the double mutant was 273 ($\pm$100) compared to 329 ($\pm$105) for the parental Hmu^+ Has^+ strain. Thus we have no evidence that hemin uptake systems play a role in the pathogenesis of bubonic or pneumonic plague.

Despite this lack of an in vivo phenotype for hemin utilization, the Hmu system is important for the intracellular growth of *Y. pestis* KIM in J77A.1 cells. During the first 9–12 h after uptake into the J774A.1 cells, there is a killing phase that is similar in both the Hmu^+ and Hmu^- strains. While the Hmu^+ strain recovers and grows to significant intracellular levels after this, the Hmu^- mutant does not recover to the same extent (Fig. 12). This pattern in the Hmu^- mutant resembles that seen in an *feo yfe* double mutant. (Perry et al. 2007).

The Has hemophore system

The Has system has been extensively studied in enterics. This system consists of the HasA hemophore

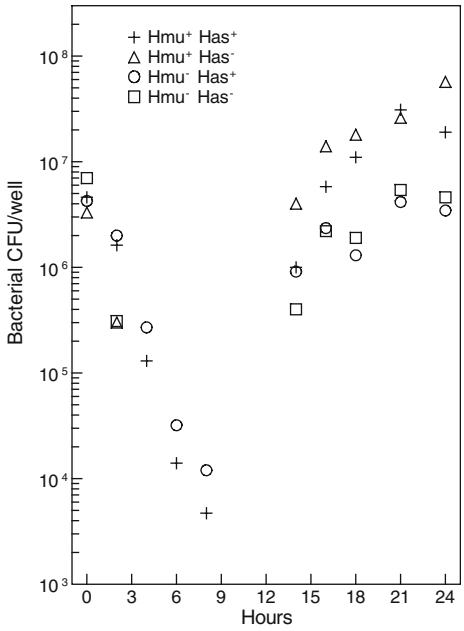


Fig. 12 Growth of *Y. pestis* strains at 37°C in cells of the murine macrophage cell line J774A.1. Duplicate wells with $\sim 4 \times 10^5$ J774A.1 cells/well were harvested for each time point. All *Y. pestis* strains used are *Δpgm* derivatives that lack the Ybt iron transport system. Pgm^+ and Ybt^- or *Δpgm* mutants exhibited similar intracellular growth in J774A.1 cells (data not shown). Results for the Hmu^+ Has^+ and Hmu^-Has^+ strains are a compilation of two or more independent experiments. Results for the Hmu^+ Has^- and Hmu^-Has^- are from one experiment that was representative of two or more independent experiments

which is secreted by HasD, HasE, and HasF/TolC. HasA can bind free hemin or remove hemin from hemoproteins and then is bound by the OM receptor HasR. HasB is a TonB-like protein that may be required for uptake of heme through the OM via the Has system (Cescau et al. 2007). All 5 *Y. pestis* genomes encode identical ORFs in all but *hasD* which has one amino acid change (and a 6-bp deletion in 91001 only; not shown in Fig. 11b) and *hasR* (with one residue change in Pestoides F only). *Y. pseudotuberculosis* IP32953 and PB1/+ have 3–5 residues changes in all 6 known components of this system. Thus there is essentially one genetic pattern for the *has* system in *Y. pestis* and *Y. pseudotuberculosis* (Fig. 11b).

Despite the production and secretion of HasA, no defect in hemin or hemoprotein utilization was found in *Y. pestis* KIM or 6/69c *has* mutants. Since the *hasR* promoter is transcribed under iron-deficient conditions and HasA binds hemin, lack of expression or failure to bind hemin are not causes for the lack of a mutant phenotype. All in vitro evidence suggests that the Has system is nonfunctional in *Y. pestis* KIM and 6/69c (Rossi et al. 2001).

In vivo analyses have also failed to identify a function for the *Y. pestis* Has system. As described above, a *Y. pestis* KIM *hmu has* double mutant was fully virulent in mouse models of bubonic and pneumonic plague (this study; Rossi et al. 2001). Since an *hmu* mutant did exhibit a loss of intracellular growth in J774A.1 cells (see above), we tested the growth of *has* mutants in this cell line. A *Y. pestis* KIM *has* mutant showed no growth defect while the *hmu has* double mutant had a growth defect essentially identical to that of the *hmu* mutant (Fig. 12).

Discussion

We initiated this study to analyze proven and putative iron transport systems encoded in the *Y. pestis* KIM genome and compare these to systems encoded by other *Y. pestis* biotypes and *Y. pseudotuberculosis*. We have identified 16 potential iron transport systems in *Y. pestis* KIM. Of these, 6 have been experimentally proven to be fully functional—Ybt, Yfe, Yfu, Yiu, Feo, and Hmu (Kirillina et al. 2006; Perry et al. 2004, 2007; Perry and Fetherston 2004). The Iuc system is defective for biosynthesis of aerobactin but capable of transporting both exogenously provided aerobactin or ferrichrome using the Fhu ABC transporter and OM receptors IutA or FcuA in *Y. pestis* KIM (Forman et al. 2007). The Fit, Efe, Fet, and FieF systems appear to be intact but are untested. The Has hemophore system also appears intact but experimental studies to date indicate it is nonfunctional in vitro and plays no role in mammalian pathogenesis (this study; Rossi et al. 2003). It is intriguing that the *hmu* locus is functional in vitro and highly conserved among the *Yersinia.* Although *Y. pestis hmu* mutants are unable to utilize hemin sources and show a defect in intracellular growth in J774A.1 cells (Fig. 12), this system appears to play no significant role in mammalian disease. A *Y. pestis* KIM *hmu has* double mutant was fully virulent in mouse models of bubonic and pneumonic plague (this study; Rossi et al. 2003). The Ynp, Ysu, and Fiu system have either been disrupted by an IS*100* element or a frameshift mutation in most if not all *Y. pestis* strains but

appear to be intact in the *Y. pseudotuberculosis* strains that were examined.

Overall, our comparisons across strains and species showed a high degree of similarity (Table 2). Seven of the iron transport systems had one genetic pattern and were nearly identical in all predicted protein residues for all 7 genomes analyzed. Five of these (Ybt, Yfe, Yfu, Yiu, Hmu) have been shown to be functional in *Y. pestis* KIM (Kirillina et al. 2006; Perry et al. 2004, 2007; Perry and Fetherston 2004); the sixth and seventh, Fit and FieF, have not been tested in *Yersinia*. Fhu and Feo systems (both functional in *Y. pestis* KIM) as well as the untested Efe and Fet systems exhibit two patterns (Table 2). The Has system, which seems to be functionally defective (Rossi et al. 2001), also exhibits two genetic patterns. Putative systems exhibiting genetic variation are usually disrupted by a frameshift mutation or an IS*100* element—Ynp, Ysu, Fiu, Iuc, Efe, and Fet. The *ynp* locus has 5 defective genetic patterns in the 5 *Y. pestis* strains analyzed, but is apparently intact and possibly functional in the two *Y. pseudotuberculosis* strains. The Fiu, Efe, and Fet (all with two genetic patterns; Table 2) systems are untested in *Yersinia.* The overall trend suggests that non-functional and possibly unimportant iron transport systems have two or more genetic variations while functional systems usually have one pattern.

One hypothesis was that the diverse transmission routes and disease patterns might require different iron transport systems. Our computational analysis found that epidemic strains of *Y. pestis* do not possess additional iron uptake systems. Instead the *Y. pseudotuberculosis* strains examined have apparently intact Ynp, Ysu, Iuc, and Fiu systems. Thus additional iron transport systems in epidemic strains are not involved in the different disease progression and increased lethality of these organisms compared to endemic *Y. pestis* strains and *Y. pseudotuberculosis*.

Both *ysu* and *ynp* systems are transcriptionally active and Fur regulated in *Y. pestis* 91001. In addition, the *ysu* loci appear intact in in *Y. pestis* CO92 and the endemic strain Pestoides F. However, our O-CAS assay study suggests that *Y. pestis* CO92 does not make an additional non-Ybt siderophore (Fig. 6). One unexpected finding was that CO92 appears to produce more Ybt siderophore than KIM6+ under similar growth conditions. Analysis of the sequences of *ybt* gene promoters and the Fur proteins in these two strains failed to identify differences that would account for this. One possibility is that differences in the metabolism of these organisms account for an increased iron requirement for CO92 compared to KIM6+.

In epidemic strain KIM, the Ybt and Yfe systems have a demonstrated role in the virulence of bubonic plague (Bearden et al. 1997; Bearden and Perry 1999; Fetherston et al. 1999). So far, these two systems in *Y. pseudotuberculosis* are the only iron transport systems that have been tested and found to affect the virulence of this organism (Carniel et al. 1992; Karlyshev et al. 2001; Une and Brubaker 1984). It is possible that the additional iron transport systems in *Y. pseudotuberculosis* may aid in survival and growth in the environment. If *Y. pestis* CO92 produces a Ysu siderophore, this does not seem to affect virulence in mouse models of plague—LD_{50} studies with KIM and CO92 strains exhibit similar values (Forman et al. 2008; Gong et al. 2001; Kirillina et al. 2006; Lathem et al. 2005).

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