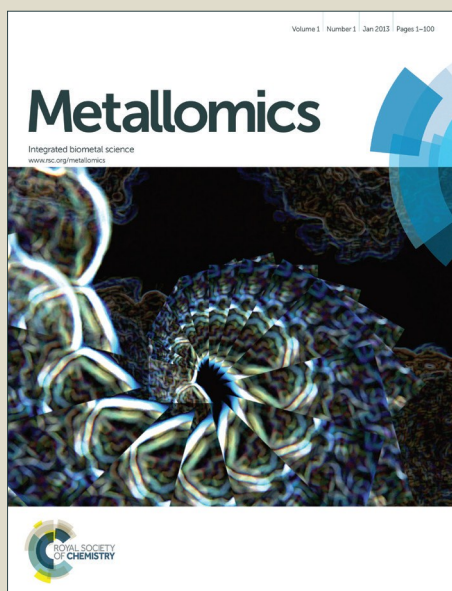


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REVIEW

The Role of Transition Metal Transporters for Iron, Zinc, Manganese, and Copper in the Pathogenesis of *Yersinia pestis*

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www.rsc.org/Robert D. Perry^a, Alexander G. Bobrov^a, and Jacqueline D. Fetherston^a

Yersinia pestis, the causative agent of bubonic, septicemic and pneumonic plague, encodes a multitude of Fe transport systems. Some of these are defective due to frameshift or IS element insertions, while others are functional *in vitro* but have no established role in causing infections. Indeed only 3 Fe transporters (Ybt, Yfe and Feo) have been shown to be important in at least one form of plague. The yersiniabactin (Ybt) system is essential in the early dermal/lymphatic stages of bubonic plague, irrelevant in the septicemic stage, and critical in pneumonic plague. Two Mn transporters have been characterized (Yfe and MntH). These two systems play a role in bubonic plague but the double *yfe mntH* mutant is fully virulent in a mouse model of pneumonic plague. The same *in vivo* phenotype occurs with a mutant lacking two (Yfe and Feo) of four ferrous transporters. A role for the Ybt siderophore in Zn acquisition has been revealed. Ybt-dependent Zn acquisition uses a transport system completely independent of the Fe-Ybt uptake system. Together Ybt components and ZnuABC play a critical role in Zn acquisition *in vivo*. Single mutants in either system retain high virulence in a mouse model of septicemic plague while the double mutant is completely avirulent.

individuals are the cause of the highly fatal primary pneumonic plague.

Two unique aspects of *Y. pestis* metal homeostasis are that each type of plague infection depends upon different metal transporters for virulence and the *in vitro* and *in vivo* phenotypes of metal transport mutants often differ.

^a Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, KY USA

Introduction

The transition metals iron, manganese and zinc play key roles in the metabolism of eukaryotes as well as bacteria. Hosts restrict access to these metals in an attempt to prevent bacterial pathogens from proliferating.¹⁻⁶ While copper serves as a cofactor in some proteins, bacteria also possess transporters and periplasmic proteins designed to avoid copper toxicity.⁷ *Yersinia pestis* has multiple, proven ferric and ferrous transport systems (Fig. 1) but only two transport systems each for manganese and zinc⁸⁻¹³ (Fig. 2) and no Cu resistance mechanism investigated. This article reviews these transport mechanisms and their roles in the virulence of *Y. pestis*.

Y. pestis causes primarily three forms of plague: bubonic, septicemic and pneumonic. Bubonic plague, caused by the bite of an infected flea, has an early lymphatic stage prior to development of systemic disease while septicemic plague bypasses the lymphatic stage. In humans, these forms of plague occasionally develop into secondary pneumonic plague. Infectious respiratory droplets transmitted from these

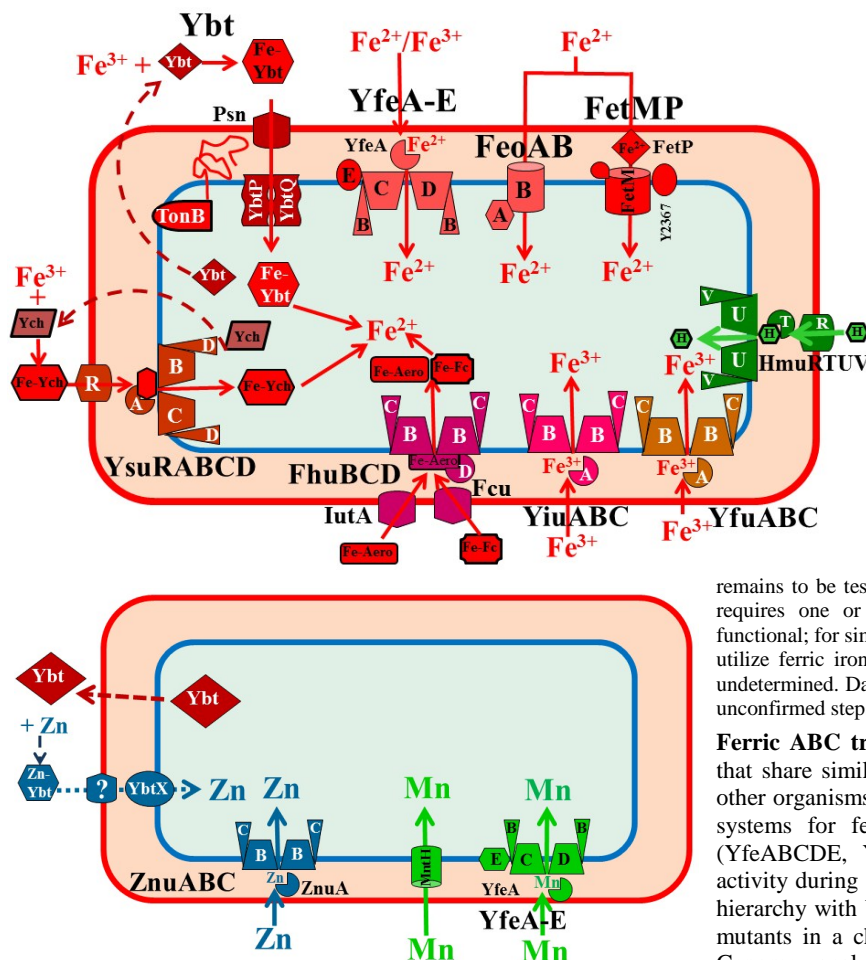
In vitro and *in silico* analysis of transition metal transporters in *Yersinia pestis*

Iron

Iron is required for the growth of nearly all bacterial species and is found in heme proteins such as catalase and cytochromes, iron-sulfur cluster (Fe/S)-containing electron carriers and enzymes, and non-heme Fe enzymes. In most cells, Fe has multiple essential roles.¹⁴

Jackson and Burrows discovered that *Y. pestis* required iron for *in vivo* growth in the 1950's. Genomic and experimental analysis has demonstrated that *Y. pestis* encodes a multitude of proven (Fig. 1), putative, and non-functional iron transport systems^{9,12} which can be divided into the four categories below. TonB/ExbB/ExbD requirements and Fur regulation of the promoters of these systems, are typical of other Gram-negative bacteria and will not be addressed here.

Heme uptake systems. The *Y. pestis* HmuP'RSTUV ABC transporter for heme and a variety of hemoproteins (Fig. 1) is typical for Gram-negatives and is nearly identical to *Yersinia enterocolitica*



negative bacteria, *in vitro* studies were unable to demonstrate transport activity for the Has hemophore system in *Y. pestis*.^{20,21}

Fig. 1. *Y. pestis* iron transport systems that are proven functional *in vitro*. These include a heme transporter (Hmu), three ferrous transporters (Yfe, Feo, and Fet), three “ferric” transporters (Yfe, Yfu, and Yiu), one siderophore-dependent system (Ybt) fully functional in all *Y. pestis* biotypes, one system (Fhu/IutA/Fcu) capable of transporting, but not synthesizing aerobactin and ferrichrome bound to Fe (FeAero and FeFc). Finally, the Ysu system of *Y. pestis* CO92 (*orientalis* biotype) synthesizes and transports the Yersiniachelin (Ych) siderophore while *Y. pestis* KIM and Antiqua (*mediaevalis* and *antiqua* biotypes) have a frameshift mutation that disrupts Ych synthesis. Although the *fitABC* and *efeUOB* loci are intact, their functionality

remains to be tested. All OM receptors shown are TonB-dependent. FetMP requires one or more of the *flp* gene products (Y2363-Y2367) to be functional; for simplicity only Y2367 is shown. The Yfe ABC transporter can utilize ferric iron as a substrate, but its oxidation state during transport is undetermined. Dashed arrows in all figures indicate unknown components or unconfirmed steps.

Ferric ABC transporters. *Y. pestis* has several ABC transporters that share similarity to ones with proven roles in Fe acquisition in other organisms. In this section only the non-siderophore-associated systems for ferric iron are discussed. Three ABC transporters (YfeABCDE, YfuABC and YiuABC) have proven iron uptake activity during aerobic growth (Fig. 1). Their efficacy has a definite hierarchy with Yfe>Yfu>Yiu, as determined by growth studies with mutants in a chelex-100 extracted, defined medium (cPMH2).²²⁻²⁵ Genome analysis identified *fitABC* as a potential Fe ABC transporter; however no experimental analysis of the system has been performed.^{9, 12} Finally, a frameshift mutation has disrupted *y2840* of the *fiuABC* locus (*y2842-y2837*) making it unlikely that this gene expresses a functional IM permease.⁹

Growth studies of a *yfe* mutant demonstrated an aerobic and microaerobic growth defect under iron-depleted conditions, implicating it in both ferric and ferrous uptake. A reduction in ferric uptake by this mutant was directly demonstrated. Thus Yfe appears to function aerobically and microaerobically (Fig. 1). Whether ferric uptake by Yfe is due to reduction to ferrous prior to transport through this system remains to be determined.

Ferrous transporters. Three (Yfe, Feo and Fet) of the four ferrous transporters of *Y. pestis* have demonstrated iron acquisition activity under microaerobic conditions.^{26, 27} The Feo, Efe and Yfe/Sit systems are widespread while the more recently discovered Fet system appears to be more narrowly distributed (Fig. 1 and 3).^{26, 28-31} The transport activities of Yfe for Fe and Mn under aerobic conditions are described elsewhere in this article.

Under microaerobic, iron-restricted conditions, the yersiniabactin (Ybt) siderophore-dependent iron transport system still plays a role in growth. This might be due to residual ferric iron from aerobic medium preparation; to avoid this complication a Δ *pgm* or *irp2* (iron-regulated protein whose gene product is required for Ybt synthesis) background was used in microaerobic and reducing conditions.^{32, 33} The *pgm* locus is a 102-kb chromosomal region that is spontaneously deleted in *Y. pestis* during *in vitro* growth and encodes the Ybt and Fet systems as well as numerous other proteins.^{9,26}

Fig. 2. *Y. pestis* manganese and zinc transporters functional *in vitro*. Two widespread Mn transporters (MntH and Yfe/Sit) are functional in *Y. pestis*. The Znu and Ybt systems are also widespread with the Ybt siderophore and YbtX recently implicated in Zn uptake. Our model shows Zn bound to Ybt. However, this has not been demonstrated and the role of Ybt in Zn acquisition may be indirect.

HemPRSTUV. HmuR is the TonB-dependent outer membrane (OM) receptor.¹⁵⁻¹⁷ The substrate-binding protein (SBP) HmuT binds two stacked hemes with nanomolar dissociation constants suggesting transport of two heme molecules in one reaction cycle.¹⁸ HmuU and HmuV are the inner membrane (IM) permease and the ATPase, respectively,¹⁷ and have a 2:2 stoichiometry. Mutational analysis of HmuU identified key amino acids for interaction with HmuT or heme transport. Analysis of the crystal structure of HmuUV showed an outward-facing cavity in the nucleotide-free complex. This suggests that this and other type II ABC transporters, which include siderophore and cobalamin transporters, may have coupling mechanisms that differ from those of other ABC transporters.¹⁹ HmuS is a homologue of the cytoplasmic heme-binding protein ShuS of *Shigella dysenteriae*.^{16,17}

A *Y. pestis* *hmuR* mutant was unable to use heme or any of the tested hemoproteins (haemoglobin, myoglobin, haemoglobin-haptoglobin, heme-albumin or hemopexin). In contrast an *hmuS* mutant was fully competent in heme/hemoprotein use.¹⁷ In *Y. enterocolitica* an *hemS* mutation is lethal.¹⁶ A *Y. pestis* *hmuT::cat* mutation prevented the use of heme and all hemoproteins except haemoglobin and hemopexin.¹⁷

Although *Y. pestis* encodes an intact hemophore system (*hasRADEB* and *hasF*) reminiscent of those found in other Gram-

Single *yfe* or *feo* mutants exhibit a significant growth defect; however a double *yfe feo* mutant has a more severe growth restriction. Complementation with either locus, supplementation with Fe, or aerobic growth alleviate this growth inhibition.^{26, 27} Further experiments demonstrated that the Fet-Flp transporter (Fig. 1 and 3) also contributes to microaerobic iron acquisition and that a *yfe feo fet* mutant is defective in short-term iron uptake under reducing conditions where the oxidation state would be primarily ferrous.²⁶

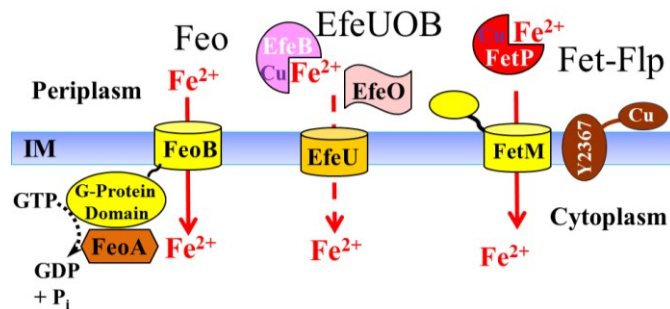


Fig. 3. Ferrous transporters of *Y. pestis*. The Yfe ABC transporter is depicted in Fig. 1 and 5. *Y. pestis* FeoAB is typical of Gram-negative Feo systems. Both FeoA and FeoB are required with FeoB proposed to serve as the permease with its G-protein domain and FeoA acting to help hydrolyze GTP to energize transport. FeoC has been proposed or shown to affect levels of FeoB,^{28, 34} however, a *Y. pestis feoC* mutation has no effect on *feoABC* transcription or growth under iron-deficient conditions.^{26, 28} Bacterial Efe systems are members of the OFeT family that are related to the yeast FTR family but lack the Fet3p component. EfeU is the permease while EfeO and EfeB are periplasmic proteins, EfeO has putative metal binding sites (Cu and Fe), an N-terminal cupridoxin domain and a C-terminal peptidase-M75 domain. EfeB is predicted to have heme peroxidase-like activity^{30, 35-37}. FetMP is distantly related to the yeast FTR system with mutations in *E. coli* and *Y. pestis* having different phenotypes (see text). In addition, an insertion in *Y. pestis y2367* (*flpD*; the first of 6 linked genes) causes loss of function. Although only Y2367 is shown in the model, the identity and number of *flp* genes (*fet*-linked phenotype) required for FetMP function is undetermined^{10, 26, 29}

Studies in *E. coli* demonstrated that FetM is an essential IM component while FetP is an accessory periplasmic component.²⁹ In contrast, a *Y. pestis fetP* mutant has a more severe growth defect than a *fetM* mutant. The FetMP system in *Y. pestis* (Fig. 3) appears to be linked to a downstream locus (*y2367-y2362*) which we have designated *flpDABCTE* (*fet*-linked phenotype). A polar mutation in *flpD* has a phenotype similar to a *fetP* mutation. FlpD (Y2367) has a partial YHS domain found in copper-transporting ATPases but lacks two cys residues that may be required for its function.^{26, 38} FlpA and B (Y2366 and Y2365, respectively) likely are ABC permeases with similarities to those predicted to export antimicrobial peptides while FlpC is the associated ATPase. FlpT has a periplasmic thioredoxin-like domain implicated in disulphide reductase activity and FlpE is a member of the cytochrome *c* superfamily.²⁶ The number and identity of genes in the *flp* locus required for iron acquisition via FetMP remains to be determined.

In other bacteria, *feo* is transcriptionally regulated by ArcA, Fnr, and RstA as well as Fur.³⁹⁻⁴² In *Salmonella* FeoC binding to FeoB resulted in higher levels of FeoB possibly by preventing degradation by FtsH.³⁴ FeoC has also been proposed as a transcriptional regulator.^{28, 43} However, our studies with an *feo* transcriptional reporter have failed to identify a role for any of these regulators in the expression of the *feoABC* operon in *Y. pestis*.²⁶

Another unique aspect of *feo* regulation in *Y. pestis* is our finding that Fe-Fur represses transcription of an *feo* reporter under microaerobic but not aerobic conditions. In contrast, all other tested iron-regulated promoters of *Y. pestis* are repressed aerobically by iron and Fur.^{12, 26, 44} The mechanism by which Fe-Fur fails to repress expression of *feo* under aerobic growth conditions is being explored.

Siderophore systems. In *Y. pestis* strains we analysed, the *ynp* locus (*y3404-y3423*) is disrupted by an *IS100* element and/or a frameshift mutation in the second gene of the locus encoding a putative siderophore biosynthetic gene or is absent entirely. In the two *Y. pseudotuberculosis* strains examined, the locus appears intact, encoding genes for siderophore biosynthesis and transport with significant similarities to the Ybt system. However, no studies on the functionality of this locus have been performed in either organism.⁹

The aerobactin system of *Y. pestis* has a frameshift mutation in *iucA* that abolishes synthesis of the siderophore. However, *Y. pestis* cells use exogenously supplied aerobactin and ferrichrome for iron acquisition via the *IutA/FhuBCD* and likely *Fcu/FhuBCD* transport systems, respectively (Fig. 1).^{9, 45}

Y. pestis strains are divided into epidemic strains (biotypes/biovars *antiqua*, *mediaevalis* and *orientalis*) that have caused epidemic/pandemics and more ancient endemic strains (Microtus or Pestoides) which cause bubonic plague in some animals and are occasionally isolated from humans.⁴⁶⁻⁴⁹ In *Y. pestis* KIM (Kurdistan Iran Man; *mediaevalis*) and Antiqua (*antiqua*) the first gene of the *ysu* locus has a frameshift mutation that should prevent synthesis of a putative hydroxamate siderophore. In *Y. pestis* CO92 (*orientalis*), 91001 (Microtus) and Pestoides F (Pestoides) as well as two *Y. pseudotuberculosis* strains, the *ysu* locus is intact (*ypo1528-y21538* in CO92) (Fig. 1). Analysis of the biosynthetic proteins encoded by the intact locus suggested two alternative biosynthetic pathways with two possible products for each pathway. The four predicted, alternative siderophore structures are all cyclic hydroxamates with estimated molecular masses ranging from ~370 to ~600 Da.⁹ Recently Rakin *et al*¹³ have demonstrated that the *ysu* locus is responsible for the synthesis of a siderophore they named yersiniachelin (Ych) in a *Y. pestis* strain with an intact *ysu* locus.

The Ybt system is encoded on a pathogenicity island and is functional in a number of different Gram-negative pathogens.⁵⁰ Early research on the Ybt system focused on *Y. enterocolitica* and *Y. pestis*. Indeed, *Y. enterocolitica* was first used to purify and determine the Ybt structure (Fig. 4) and identify synthesis of Ybt diastereomers.^{51, 52} The Yersinia Ybt system has been reviewed recently,⁵³ consequently, biosynthesis of the Ybt siderophore will not be discussed here and regulation will only be briefly summarized.

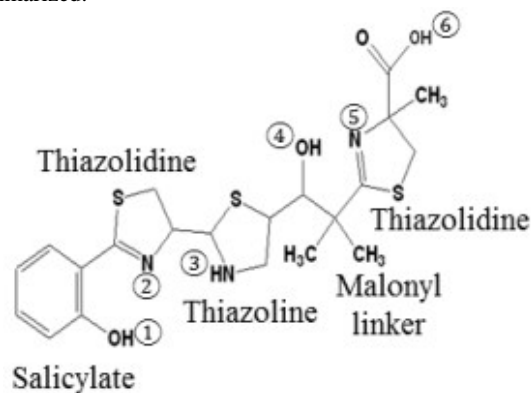


Fig. 4. Structure of the Ybt siderophore. Salicylate, thiazolidine, and thiazoline rings as well as the malonyl linker are labelled. The six coordinate binding sites for Fe^{3+} are shown.

The mechanism for secretion of Ybt has not been elucidated. Once in the environment, Ybt can remove iron from transferrin and lactoferrin under *in vitro* conditions and has a calculated proton-independent stability constant for Fe^{3+} of $4 \times 10^{36} \text{ M}^{-1}$ with three nitrogen and three hydroxyl coordination sites (Fig. 4).^{54, 55} The crystal structure of Fe^{3+} -Ybt has been characterized.⁵⁶ Although uptake of Fe from Ybt has been demonstrated, translocation of the

siderophore into the bacterial cytoplasm has not.⁵³ Uptake requires the TonB-dependent OM receptor Psn (also required for sensitivity to the bacteriocin pesticin and termed FyuA in other bacteria) and two fused-function IM permease-ATPases, YbtP and YbtQ (Fig. 1). Although this type of fused-function ABC transporter is usually associated with Type I secretion systems, *ybtP* and *ybtQ* mutants exhibit reduced iron uptake and iron-deficient growth but normal levels of secreted Ybt.⁵⁷ No SBP for this transport system has been identified and it may be that none is required. Compared to the parent strain and an *irp2* (biosynthetic) mutant, *psn*, *ybtP* and *ybtQ* mutants all show reduced iron-deficient growth. It is likely the secreted Ybt in the transport mutants sequesters residual iron making it unavailable for use by other iron transport systems. Indeed, mutations in other iron transport systems have no *in vitro* phenotype unless the Ybt system is also mutated. Where or how Fe is removed from the Ybt siderophore also remains unresolved.^{53, 55}

The function of YbtX, an IM protein with similarities to pyochelin and rhizobactin siderophore uptake permeases but also with modest similarities to alcaligin and enterobactin siderophore exporters, remained unknown until recently (see **Zinc** section below). Although encoded within the *ybtPQXS* operon, a *ybtX* mutant shows normal Ybt secretion and iron uptake. In addition, iron-deficient growth of this mutant is modestly better than the parent strain indicating that YbtX has no significant role in Fe uptake by the Ybt system.⁵⁷

The *ynp* locus (see above) has genes which encode proteins with similarities to some Ybt biosynthetic enzymes and to Psn, YbtP, YbtQ, and YbtX. However, none of these proteins correct defects in Ybt synthesis or transport due to *ybt* mutations.^{9, 53}

In addition to typical Fe-Fur repression of the four *ybt* operons encoded within the pathogenicity island, transcription is regulated by YbtA, a member of the AraC family of transcriptional regulators, and the Ybt siderophore. YbtA in the presence of Ybt activates transcription of the *irp2irp1ybtUTE*, *ybtPQXS*, and *psn* operons and represses its own transcription. All mutations that prevent Ybt synthesis show loss of transcriptional activation by YbtA with one exception. A *ybtS* mutant which cannot synthesize salicylate and had no detectable Ybt still exhibits normal YbtA-dependent transcriptional activation. The mechanism behind this anomaly is under investigation.^{33, 57-59}

Zinc

Zinc is an essential element with no known examples of organisms that have completely dispensed with a Zn requirement. Zn is not redox active under normal physiological conditions, but serves as an electrophilic catalyst (Lewis acid) in numerous enzymes and as a scaffold for organizing protein domains¹⁴. Our studies have shown a Zn requirement for *in vitro* growth of *Y. pestis*.⁶⁰

Bioinformatic analysis of the *Y. pestis* KIM10+ genome identified the typical ZnuABC transporter as the only Zn uptake system (Fig. 2) and 4 Zn efflux systems (*ZntA*, *ZitB*, *ZntB*, and *FieF*).⁶⁰ No experimental work has been published on Zn efflux in *Y. pestis* and Zn-Zur regulation is typical of other Gram-negative bacteria and will not be discussed here.^{60, 61}

Compared to the parent strain, a *Y. pestis znu* mutant had a significant growth defect in cPMH2 (residual Zn content of ~0.5 μ M) which is corrected by supplementation with Zn but not Fe and by complementation with the cloned *znuABC* locus. However, the *znu* mutant responded to the addition of Zn to 0.4 μ M suggesting the presence of an additional high-affinity Zn uptake system.⁶⁰

In our search for this putative Zn transporter, we mutated or overexpressed other divalent cation transporters, transporters whose expression is Zn-regulated and the homolog of ZevAB, a more recently identified Zn transporter of *Haemophilus influenzae*.⁶² We also tested the growth of a septuplet mutant (*znu yfe mntH efe fetMP*

feo y2842) in case there are three or more semi-redundant Zn transporters in *Y. pestis*. None of these studies indicated a role in Zn acquisition for any of these systems, at least under our *in vitro* Zn-deficient growth conditions. Thus the ferrous transporters (Yfe, Feo, Efe, Fet), manganese uptake systems (MntH and Yfe), ZevAB, Y2842 (a Zn-responsive SBP named FiuA), Zn-responsive putative K-channel Y1247, and FieF appear to play no role in Zn acquisition in *Y. pestis* (unpublished observations).^{8, 10, 60, 62} Although FieF is a Zn and Fe effluxer in bacteria, human and plant homologues of FieF appear to import Zn.⁶³⁻⁶⁵

Since Ybt binds another divalent cation (Cu^{2+}),⁶⁶ we constructed an *irp2 znu* mutant and found that this double mutant, unable to synthesize Ybt, was unable to grow in cPMH2 supplemented with 1 μ M Fe (to compensate for loss of the Ybt system) and 0.6 μ M Zn. Growth was restored by complementation with the cloned *irp2* gene, supplementation with 2.5 μ M Zn or addition of purified Ybt siderophore. These results suggest that the Ybt siderophore is involved in high-affinity Zn acquisition and may serve as a zincophore. However, we have not demonstrated Zn binding by Ybt.⁸ Pyoverdine and pyochelin bind a variety of divalent and trivalent cations but these non-Fe complexes appear to be involved in preventing metal toxicities.⁶⁷⁻⁶⁹ Both pyochelin and micacocidin (an anti-mycoplasmal compound produced by a *Pseudomonas* and *Ralstonia solanacearum*) bind Zn and have structures similar to Ybt.^{67, 70, 71} In *R. solanacearum*, Kreutzer *et al* used gallium as a surrogate for Fe to demonstrate potential siderophore activity by micacocidin.⁷¹ Coelibactin, produced by *Streptomyces coelicolor* has been proposed as a zincophore based on Zn and Zur regulation of the coelibactin locus.^{72, 73} However, neither Zn binding nor uptake via coelibactin has been demonstrated. The only other secondary metabolite that has been shown to both bind Zn and deliver it to the bacterial cell is pyridine-2,6-bis(thiocarboxylic acid) (PDTC) produced by some *Pseudomonas*. Although PDTC is classified as a siderophore and delivers Fe more effectively than Zn to the cell, it may serve to reduce Fe to the ferrous state.^{74, 75}

To identify possible Ybt transport components involved in Zn acquisition, we assessed the Zn-deficient growth of *psn*, *tonB*, *ybtP*, *ybtQ* and *ybtX* mutants in a *znu* background. These studies demonstrated that Psn, TonB, YbtP and YbtQ, required for Fe uptake via Ybt, are not involved in Zn acquisition. Instead, the IM permease YbtX is required for Zn uptake via Ybt (Fig. 2).⁸ This is the first siderophore system with demonstrated involvement in Fe and Zn acquisition by completely independent uptake systems.

Manganese

Y. pestis has two identified Mn transporters that are widespread in bacteria (Fig. 5). MntH, an Nramp1 family member, was first characterized in *Salmonella* and *E. coli* as a proton-dependent Mn transporter. The *Y. pestis* MntH is highly similar to other characterized MntH transporters involved in Mn uptake.^{11, 64, 65}

ABC transporters for Mn were first described in the cyanobacterium *Synechocystis*, Streptococci and then *Y. pestis*.^{22, 23, 76-78} In *Y. pestis*, it was cloned in a screen for iron transport systems and named Yfe; however, Yfe transports both Fe and Mn.^{22, 23} Subsequently Gram-negative orthologues were identified in *Salmonella* and *Shigella* and termed Sit.⁷⁹⁻⁸¹ While YfeA-D is a typical ABC Mn transporter,^{6, 22, 23} the SBP YfeA binds Mn and Zn with similar high affinities (18 nM and 7 nM, respectively) yet transports only Fe and Mn.⁶⁰ The PsaA SBP component of the Mn ABC transporter of *Streptococcus pneumoniae* (PsaABC) also binds both Mn and Zn but transports only Mn. Couñago *et al* have shown that Zn binding by PsaA locks it in a closed state that prevents release of the cation;⁸² perhaps a similar mechanism occurs with the related YfeA of *Y. pestis*. In *Y. pestis*, there is an additional IM component, YfeE (Fig. 5). In cPMH supplemented with conalbumin

or 2,2'-dipyridyl (DIP), a *Y. pestis* *ΔyfeE* mutant had a moderate delay in growth while *ΔyfeA*, *ΔyfeAB* and *ΔyfeABCDE* mutants all showed significant growth inhibition.^{22, 23} The role of YfeE in Fe or Mn transport remains to be determined. In unchelated cPMH2 (containing ~0.5 μM residual Mn), single *mntH* and *yfe* *Y. pestis* mutants have no significant growth defect, while the *mntH yfeAB* double mutant has a modest growth defect. This double mutant also shows complete loss of Mn uptake in short-term (30 min) transport assays.¹¹

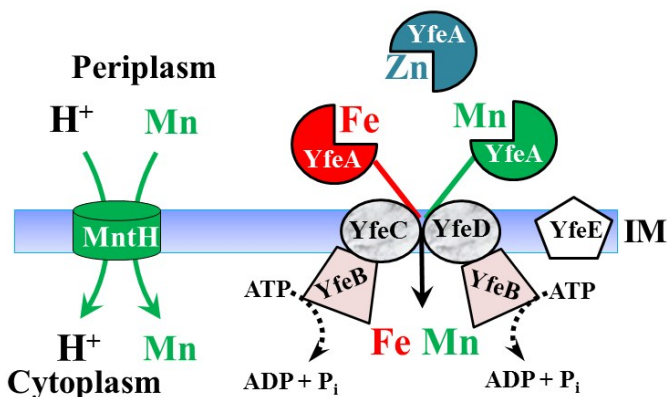


Fig. 5. Models of proven *Y. pestis* Mn transporters YfeABCDE and MntH. The Yfe ABC transporter also accumulated Fe under aerobic and microaerobic conditions. In addition, the YfeA SBP binds Zn but does not transport it into the cytoplasm.^{22, 23, 26, 60}

The modest growth defect of this double mutant and a growth response to micromolar Mn suggests that a third, unrecognized high-affinity Mn transporter may provide Mn. Our initial tests of a quintuplet mutant (*yfe mntH feo fet efe*) and septuplet mutant (with additional *znu y2842* mutations) indicate that none of these systems are the putative 3rd Mn transporter. Indeed, the septuplet mutant responds to Mn supplementation to 1 μM.¹¹ While this supports a putative 3rd Mn transporter, the modest *in vitro* growth defect may simply reflect little or no requirement for Mn by *Y. pestis*. Indeed there is significant evidence that *E. coli* does not require Mn but can use Fe to replace Mn in some mononuclear enzymes and has Fe-dependent isozymes.^{83, 84} One example is ribonucleotide reductase (RNR); *Y. pestis*, *E. coli* and other enterics have two Fe-dependent RNRs and one Mn-dependent RNR. In contrast, *Bacillus subtilis* has a single Mn-dependent RNR and appears to have an absolute requirement for Mn.^{14, 85, 86} The ability of Fe supplementation to 1 μM to stimulate growth of our *yfe mntH* mutant supports the view that *Y. pestis* does not have an essential requirement for Mn, at least under our *in vitro* growth conditions. The modest *in vitro* growth defect of the double mutant under Fe- and Mn-deficient growth conditions may result from diversion of scarce Fe into some normally Mn-dependent enzymes and mismetallation of some mononuclear enzymes by Zn, preventing enzymatic activity.^{84, 87, 88}

Nothing is known about the translocation of Mn through the *Y. pestis* OM. Although an OM porin (MnoP) for Mn uptake has been described for *Bradyrhizobium japonicum*,⁸⁹ no protein with significant similarity to MnoP is encoded by the *Y. pestis* genome.

A unique aspect of Mn-responsive regulation in *Y. pestis* is the lack of MntR or Mur transcriptional regulators. Instead Fe-Fur and Mn-Fur repress transcription of the *mntH* and *yfeABCD* promoters (~7-10-fold and 2-fold repression by Fe and Mn, respectively). All other tested *Y. pestis* Fe-Fur repressible promoters are not repressed by Mn supplementation. In addition, Mn repression of these two promoters is not a unique feature of the *Y. pestis* Fur protein – *E. coli* Fur causes similar Mn repression of these *Y. pestis* promoters. A fifteen nucleotide segment that partially overlaps the Fur box of the *yfeABCD* promoter displays significant similarity to a region located

upstream of the Fur box in the *mntH* promoter. When these nucleotides (catcgctaattggtat) replace similarly positioned nucleotides of the *hmuP'R* promoter, the hybrid promoter is converted from repression by Fe only to repression by Fe and Mn.^{10, 11, 23} While all other tested Fe-Fur responsive promoters do not respond to Mn,¹⁰ it is likely that any additional *Y. pestis* promoters that respond to Mn will also be repressed by Fe.

In other bacteria, *mntH* is also regulated by OxyR while *yfe* is regulated by a LysR-type transcriptional regulator (YPTB0333) in *Yersinia pseudotuberculosis*.^{81, 90, 91} In *Y. pestis*, regulation of *yfe* by the LysR-type homologue and *mntH* by OxyR has not been tested.

Copper

While copper is required for growth in a number of bacteria and serves as a cofactor in cellular processes,¹⁴ excess Cu is toxic. Relatively few Cu import systems have been identified – with P-type ATPases in *Pseudomonas aeruginosa* and *Listeria monocytogenes* being two exceptions.^{92, 93} Cu resistance systems have been characterized in a number of bacteria.⁷ In *Y. pestis* no experimental work on these systems has been performed. Our search of the *Y. pestis* KIM10+ genome did not identify a P-type ATPase Cu importer. An *in silico* analysis of 14 copper homeostasis proteins identified 7 (CopA, CueO, CueP, CusC, CutF [NlpE], PcoC and YebZ [PcoD]) which are prevalent in *Yersinia* species.⁷ Our search of the *Y. pestis* KIM10+ genome identified an additional 3 genes encoding potential copper homeostasis proteins. CueR is a transcriptional regulator controlling expression of *cueO* and *copA* in other enterics, while Y2522 and Y2523 have similarities to CopD/PcoD (35% identity/42% similarity) and CopC/PcoC (28% identity/48% similarity) in *Pseudomonas syringae*, respectively. PcoC and PcoD may import copper into the cytoplasm. Periplasmic PcoC binds Cu⁺ and Cu²⁺ and may interact with membrane protein PcoD (YebZ) (Fig. 6).⁷ Based on bioinformatics and functions characterized in other bacteria, Fig. 6 shows our proposed model of copper homeostasis in *Y. pestis*. CopA may export Cu⁺ from the cytoplasm to the periplasm. In the periplasm CueP could be a Cu-binding protein involved in copper resistance in *Salmonella*⁸⁴ while CueO appears to be a multi-copper oxidase that converts Cu⁺ to Cu²⁺.⁷ In *E. coli*, CutF is an OM protein involved in copper tolerance while CusC is the OM component of the CusCBAF complex that exports Cu⁺ from the cytoplasm to the extracellular environment.⁷ Whether CusC is capable of Cu⁺ export in the absence of the IM and periplasmic components of the system is unknown.

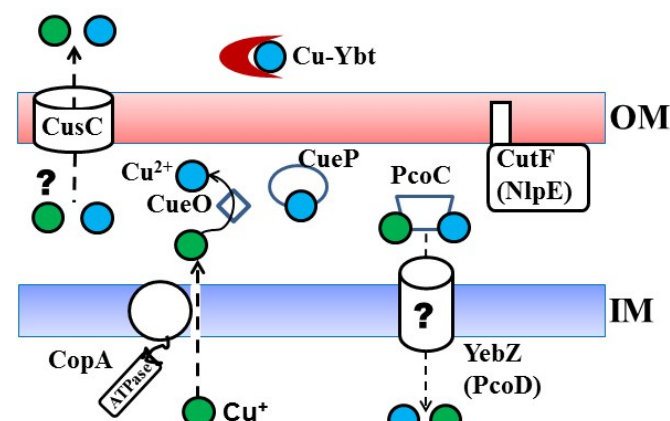


Fig. 6. Proposed *Y. pestis* copper transport and resistance mechanisms. Proposed functions for *Y. pestis* proteins are based solely on similarities to those with established functions in other bacteria. Cu²⁺ binding by the Ybt siderophore has been experimentally demonstrated.⁶⁶ Cu⁺, green spheres; Cu²⁺, blue spheres.

In *E. coli*, the Ybt siderophore has been shown to bind Cu²⁺, enhancing copper tolerance. In addition, Cu-Ybt acts as a superoxide dismutase.^{66, 94}

Ybt enhances intracellular survival of *E. coli* in copper-replete RAW264.7 cells suggesting that Cu²⁺ binding and/or superoxide dismutase activity may play a role in virulence.⁹⁴ Copper resistance genes have been associated with higher expression and/or increased survival of bacterial pathogens *in vivo*.^{84, 94-97} Microarrays analysis found that *Y. pestis cueO* was expressed in the rat bubo but not in the flea while *cueR* was upregulated 4.5-fold in the bubo compared to the flea. However, no differential expression of any other copper resistance genes was noted.⁹⁸

In vivo* roles of transition metal transporters in *Y. pestis

Y. pestis is an obligate pathogen with a life cycle alternating between growth in its vector (fleas) and mammalian hosts, primarily rodents. This section will describe our current knowledge of the transition metal transporters required for growth in the flea and for the progression of the three primary forms of plague – bubonic, septicemic and pneumonic as well as expression of these transporters in the diverse environments of the flea and different mammalian organs.

Fleas

Upon ingestion of an infected blood meal, *Y. pestis* migrates from the foregut, consisting of an unbranched esophagus and proventriculus (a valve separating the esophagus and the midgut), into the midgut. In the midgut, blood is incompletely digested by various degradative enzymes and moved to the hindgut for excretion. *Y. pestis* is restricted to this simple alimentary tract. In the classic plague vector, the oriental rat flea (*Xenopsylla cheopis*), *Y. pestis* produces the Hms biofilm in the flea gut and proventriculus that is essential for blockage-dependent transmission of *Y. pestis* from an individual flea to mammals. Biofilm formation is thought to be important for spread and long-term maintenance in natural reservoirs.⁹⁸⁻¹⁰² Early biofilm-dependent blockage of some fleas and enhanced transmission by laboratory-reared fleas exposed to natural environments suggests that blockage-dependent transmission may also play a large role in epidemics.^{103, 104} A second proven transmission mechanisms, early-phase transmission, which occurs within 24-72 h of an infectious blood meal, is proposed to play an important role in transmission from multiple flea bites and during fast-progressing plague epizootics in prairie dogs.^{100, 104, 105} One study suggests that early-phase transmission is biofilm-independent.¹⁰¹

Although no experimental studies on requirements for transition metal transporters in fleas have been published, a microarray study has yielded insights into their expression. A comparison of the transcriptomes of *Y. pestis* cells from the flea midgut to those from rat lymph nodes (buboes) revealed that no iron transport systems are highly expressed in the flea.^{98, 106} This presumed iron-rich environment might result from the lysis of red blood cells releasing haemoglobin and the degradation of various serum proteins that chelate Fe and hemin. Although *yfe* levels were higher in the bubo compared to the flea, *mntH* levels were similar in both environments.^{98, 106} Since both *yfe* and *mntH* are similarly regulated by Fe and Mn availability, this difference might be due to additional regulation of *yfe* and/or *mntH* or to technical difficulties. The flea mid-gut may be somewhat Zn-restrictive since expression levels of *rpmJ* (*ypo3135*; L36 ribosomal subunit protein) and *ypo1343* (*y2842*; FiuA), both highly Zn-repressed *in vitro*,⁶¹ were similar in the bubo and flea (see Bubonic plague below). Finally, in the flea,

there was evidence for expression of genes encoding proteins predicted to be involved in copper resistance mechanisms.^{98, 106}

Bubonic Plague

Relatively small numbers of *Y. pestis* cells (~100) are inoculated into the dermis from the infected flea bite.¹⁰⁷ The first stage of infection is growth at the bite site and dissemination to the draining lymph node. This early stage is proposed to have an intracellular phase since the *Y. pestis* antiphagocytic mechanisms may not be fully induced. Growth in the lymph node is largely extracellular, at least at the later stages, and is characterized by a swollen lymph node or bubo. Finally bacteria disseminate via the bloodstream to internal organs (liver, spleen, lungs, etc.) where they grow to high populations and eventually cause septic shock. Symptoms of bubonic plague are a sudden onset of fever, headache, chills, malaise, regional lymphadenopathy (swollen lymph node) and sepsis. Untreated, the fatality rate is 50-60% in humans. In rodents, a high level of bacteria in the bloodstream is a requirement for transmission to a naïve flea, which completes the natural life cycle of *Y. pestis*. Humans are dead end hosts and do not contribute to the maintenance of *Y. pestis* in natural reservoirs.^{61, 98-100, 108, 109}

Sebbane *et al.* did a microarray analysis of strain 195P cells isolated 48-72 h post-infection from lymph nodes compared to cells grown in a Luria Bertani/MOPS medium (LB/MOPS) at 37°C.¹¹⁰ Fur-regulated genes were generally derepressed with 120-fold increases in some *ybt* operons/genomes. However, neither *iutA* (needed for aerobactin use) nor *feoAB* were differentially regulated (Table 1).¹¹¹ Our finding that *feoABC* is not repressed by Fe under aerobic conditions *in vitro*²⁶ likely explains the lack of increased *feoABC* expression *in vivo*. Both Mn- and Fe-regulated *mntH* and *yfeA* were more highly expressed in the bubo compared to *in vitro* as were Zn-regulated *znuA* and *rpmJ* (Table 1).¹¹⁰ These results suggest that the infected bubo is an environment low in Fe, Mn, and Zn.

Long before technology allowed researchers to determine that *Y. pestis* Pgm⁻ mutants (unable to adsorb hemin, a characteristic of the Hms biofilm) result from a 102-kb chromosomal deletion, Jackson and Burrows¹¹² determined that Pgm⁻ mutants were avirulent in mice by an intraperitoneal route unless hemin or inorganic iron was co-injected. A KIM Δ *pgm* mutant is also avirulent in mice via subcutaneous injection (sc).¹¹³ Our mouse studies, using KIM strains, have shown that specific *ybt* mutations cause a similar loss of virulence by an sc route. Note that mutations in biosynthetic- and transport-encoding genes cause a similar degree of virulence loss (Table 2).^{8, 55} *In vivo* studies with strains that possess intact *ysu* and *yhc* loci (e.g., CO92 and endemic strains), show a similar virulence loss due to *ybt* mutations. This suggests that the Ysu/Yhc siderophore-dependent transport system does not compensate for the loss of Ybt *in vivo*.^{46, 114} Mutations in the Hmu and Has transporters or Yfu and Yiu do not affect virulence (Table 2).^{20, 25}

A single *feoB* mutant is fully virulent while three trials of the single *yfeAB* mutant show an 8-9-fold loss of virulence compared to the parent strain. While statistics currently indicate this difference is not significant, further mouse studies could change this calculation. However, a *feoB yfeAB* double mutant has an 89-fold loss of virulence compared to the parent strain (Table 2).²⁶ Thus the Ybt-siderophore dependent system is essential for the progression of bubonic plague while the Feo and Yfe transporters play a lesser role. An open question is whether a strain mutated in all 4 ferrous transporters would have reduced virulence compared to the Δ *feoB \Delta**yfeAB* double mutant.

Using *Y. pestis* CO92 in a Brown-Norway rat bubonic plague model, Pradel *et al.* found that single mutations in most Fe transport systems did not affect virulence. These Fe uptake systems included

Metallicomics

Table 1. Microarray analysis of *Y. pestis* gene expression under *in vivo* conditions

Gene ^a	Bubonic plague ^b	“Septicemic plague” ^b	Pneumonic plague ^b
Fe-responsive genes			
<i>irp2</i> (ypo1911)	197.3	7.1	2.1
<i>ybtP</i> (ypo1913)	B	4	3.3
<i>ybtX</i> (ypo1915)	97.8	2.9	3.1
<i>psn</i> (ypo1906)	179.7	2.5	2.3
<i>ybtA</i> (ypo1912)	24.2	1.4	-
<i>iucA</i> (ypo0989)	B	2.1	-
<i>ysul</i> (ypo1530)	4.5	1.3	-
<i>ysuF</i> (ypo1528)	11	3.1	-
<i>ysuH</i> (ypo1531)	5	1.2	-
<i>ysuR</i> (ypo1537)	-	3.3	-
<i>ysuE</i> (ypo1538)	-	6.1	-
<i>yfuA</i> (ypo2958)	18.7	3	-
<i>yiuA</i> (ypo1310)	12.6	1.7	-
<i>yiuR</i> (ypo1313)	11.9	1.5	-
<i>feoB</i> (ypo0132)	-	1.1	-2.8
<i>feoA</i> (ypo0133)	-	1.1	-3.2
<i>efeU</i> (ypo1854)	5.5	1.9	-
<i>fetM</i> (ypo1941)	11.8	-	2.2
<i>flpD</i> (y2367/ypo1943)	-	-	2.2
<i>flpA</i> (y2366 /ypo1944)	7.1	-	-
<i>hmuR/YPO0283</i>	10.7	2	-
<i>tonB</i> (ypo2193)	5.4	2.6	-
<i>exbD</i> (ypo0683)	12.1	2.5	-
Mn- and Fe-responsive genes			
<i>mntH</i> (ypo2982)	8.5	3	-
<i>yfeA</i> (ypo2439)	9.6	10.2	2.8
Zn-responsive genes			
<i>znuA</i> (ypo2061)	3.2	2.2	2.1
<i>rpnJ</i> (ypo3135)	22.7	-	23.5
<i>fiuA</i> (ypo1343)	-	1.2	28.1
<i>zntA</i> (ypo3820)	-	-	-5.4
Cu-responsive genes			
<i>pcoC</i> (y2523/ypo1784)	-	3.6	-

^a Some are the first gene of an operon. The *ypo* numbers are locus tags for *Y. pestis* CO92, referenced in the microarray studies; *y* numbers are locus tags for *Y. pestis* KIM10+. Positive numbers indicated fold increases in expression *in vivo* vs *in vitro*. Dashes indicate that the gene was not listed in the table of differentially regulated genes which could indicate no detected expression or no significant difference between the two environments. B – detected only in cells from the bubo.

^b Bubonic plague – cells from rat bubo vs log-phase cells grown in Luria Bertani/MOPS broth at 37°C¹¹¹; “septicemic plague” – cells grown in heat-inactivated human serum vs cells grown in Luria Bertani broth at 37°C^{86, 115}; pneumonic plague – cells from bronchoalveolar lavage 48h post infection vs cells grown in Brain Heart Infusion broth + 2.5 mM CaCl₂ at 37°C¹¹⁶

Efe, FcuA, Fit, Fiu, Hmu, Iuc, IutA, Yiu, Yfu, Ynp and Ysu.¹¹⁷ However, with potentially redundant siderophore, ferric and ferrous transporters, multiple mutations are often required to identify the virulence roles of these systems.²⁶

Our *znu* mutants retain high virulence (Table 2) and Pradel *et al.* obtained a similar result in Brown-Norway rats.^{60, 117} Because an *irp2* mutant is completely avirulent in the bubonic plague model, we did not test the *irp2 znu* mutant that is more severely defective in Zn acquisition *in vitro*. Since a *ybtX* mutation does not affect Fe uptake via Ybt, we are testing the virulence of a *znu ybtX* mutant to assess the role of Zn acquisition in the progression of bubonic plague.

Mn acquisition also plays a role in bubonic plague. While an *mntH* mutant remains highly virulent in both mouse and rat models, our *yfeAB mntH* double mutant has a 133-fold virulence loss

Table 2. Virulence of *Y. pestis* with Fe, Mn, or Zn mutations in mouse models of bubonic, septicemic, and pneumonic plague.

Strain or mutation (Function)	Bubonic plague LD ₅₀ ^a	Septicemic plague LD ₅₀ ^a	Pneumonic plague LD ₅₀ ^a	Reference
Parent strain	25	<14	329	8, 55
Fe transport mutants				
<i>psn</i> (Fe ³⁺ -Ybt uptake)	>2.6 x 10 ⁷ (>1.13 x 10 ⁶ -fold)	<14	1.1 x 10 ⁴ (33-fold)	8, 55
<i>irp2</i> (Ybt synthesis)	>1.3 x 10 ⁷ (>5.2 x 10 ⁵ -fold)	~48	2.6 x 10 ⁵ (790-fold)	8, 55
<i>AfeoB</i> (Fe ²⁺ uptake)	55 (NS)	Not tested	211 (NS)	26
<i>ΔyfeAB</i> (Fe & Mn uptake)	205 (9-fold; NS) 74.3 (8-fold)	Not tested	139 (NS)	24, 26
<i>ΔyfeAB AfeoB</i> (Fe ²⁺ & Mn uptake)	2,035 (89-fold)	Not tested	407 (NS)	26
<i>Δpgm ΔyfeAB</i> (Ybt Fet-Flp ⁺ ; Fe ³⁺ -Ybt & Fe ²⁺ uptake)	> 10 ⁷	< 6 (NS) ^b	>3.9 x 10 ⁶ (>1.2 x 10 ⁴ -fold)	8, 22, 55
<i>Δpgm ΔyfeAB</i> (Ybt Fet-Flp ⁺ ; Fe ³⁺ & Fe ²⁺ uptake)	Not tested	> 1.7 x 10 ^{7b} (>1.2 x 10 ⁶ -fold)	Not tested	22
<i>ΔhmuP'RSTUV</i> <i>ΔhasRADE</i> (heme uptake)	<4.2 (NS)	Not tested	273 (NS) ^c	20 (unpublished)
Mn transport mutants				
<i>ΔmntH</i> (Mn uptake)	36 (delayed TTD)	Not tested	Not tested	11
<i>ΔyfeAB ΔmntH</i> (Mn & Fe uptake)	3,068 (133-fold)	Not tested	142 (NS)	11
Zn transport mutants				
<i>znu</i> (Zn uptake)	266 ± 149 (NS) ^d	~11 ± 5	1,113 ± 1,376 (NS) ^d	8, 60
<i>psn ΔznuBC</i> (Fe ³⁺ -Ybt & Zn uptake)	Not tested	~40 (delayed TTD)	Not tested	8
<i>irp2 znu</i> (Ybt synthesis & Zn uptake)	Not tested	~6.0x 10 ⁶ (>4.3 x 10 ⁵ -fold)	Not tested	8
<i>Δpgm znu</i> (Ybt Fet-Flp ⁺ ; Fe ³⁺ , Fe ²⁺ & Zn uptake)	Not tested	5.7 x 10 ⁶ (>9.5 x 10 ⁵ -fold)	Not tested	8

^a All studies used outbred Swiss Webster mice. Bubonic plague – subcutaneous injection; Septicemic plague – retro-orbital injection; Pneumonic plague – intranasal instillation; delayed TTD – delayed time-to-death at low doses compared to parent/wild-type strain except for the *psn ΔznuBC* mutant which was compared to the *znu* mutant. NS – not significantly different from the wild-type strain.

^b The *Δpgm* and *Δpgm ΔyfeAB* mutants have a *jopJ::Mud Apsa* background. A combination of *ΔyfeAB* and genes encoded within the *pgm* locus could contribute to the large virulence loss in the *Δpgm ΔyfeAB* mutant.

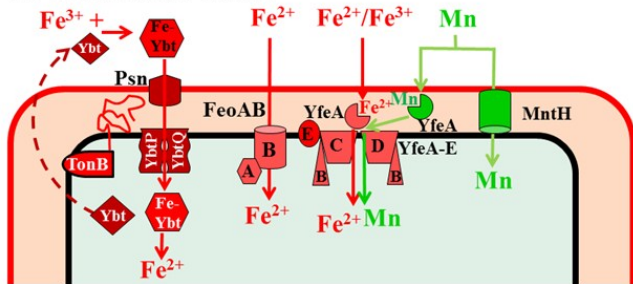
^c Unpublished data – Swiss Webster mice were infected by intranasal instillation with *Y. pestis* cells grown as described in other studies on pneumonic plague. All mouse experiments in Table 2 were conducted in accordance with the Animal Welfare Act and approved by the University of Kentucky Institutional Animal Care and Use Committee.

^d Standard deviations are shown only for the *znu* mutant because of high variations. For clarity, standard deviations are not shown for all other mutants tested.

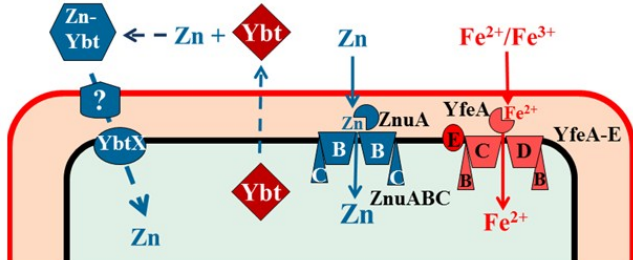
compared to the parent strain in the mouse model (Table 2).^{11, 117} We propose that the decreased virulence of the double mutant, compared to its modest *in vitro* growth defect, may be due to impaired oxidative stress defences. Although Mn is not essential for growth of *E. coli*, MntH is required during peroxide stress and Mn has been implicated in oxidative defences.^{4, 83}

Thus full virulence in a mouse model of bubonic plague requires Ybt and Yfe or Feo for Fe acquisition and Yfe or MntH for Mn acquisition with only a mutation in the Ybt system causing a potentially complete loss of virulence (Fig. 7A; Table 2).

A. Bubonic Plague



B. Septicemic Plague



C. Pneumonic Plague

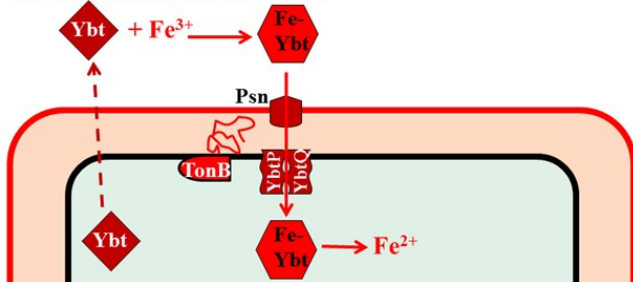


Fig. 7. Fe, Zn, and Mn transporters with proven roles in mouse models of bubonic (A), septicemic (B), and pneumonic (C) plague. The Yfe system is shown as “transporting” both Fe³⁺ and Fe²⁺ since there is evidence it functions under aerobic conditions where Fe³⁺ would predominate and under microaerobic or reducing conditions where Fe²⁺ would be prevalent. However, the oxidation state transported is undetermined. Zn binding by Ybt (B) has not been shown; thus Ybt could play an indirect role in Zn uptake.

Septicemic Plague

Septicemic plague, which is less common, has been attributed to cutaneous cuts that allow direct entry to the blood stream.^{100, 109} Recently, Sebbane *et al.*¹¹⁸ demonstrated that bites from multiple infected oriental rat fleas (*Xenopsylla cheopis*, a classical plague vector) could, at low incidence, cause primary septicemic plague. Septicemic plague is essentially bubonic plague without the early dermal and lymphatic stages and is characterized by fever and sepsis without regional lymphadenopathy (buboes)^{100, 109}.

Gene expression studies on *Y. pestis* cells isolated from the liver and spleen have not been performed. A microarray study using cells cultured *ex vivo* in heat-inactivated human plasma is the only genome-wide gene expression study relevant to septicemic plague

and the latter stages of bubonic plague. Although most Fe transporters were more highly expressed in plasma compared to growth in LB at 37°C, *iutA*, *fhuC*, *feoAB* and *fet-flp* were not differentially expressed (Table 1).^{86, 115} Both Mn- and Fe-regulated systems (MntH and Yfe) had a 3-10.2-fold increase in expression in plasma compared to LB. Expression of genes involved in Zn and Cu transport or resistance was variable. Expression of *znuA* was moderately increased in plasma but other highly Zn-repressed genes (*rpmJ* and *fiuA*) were not. Finally, *pcoC* showed moderately higher expression in plasma compared to LB while all other Cu-related genes did not (Table 1).^{86, 115} Perhaps additional non-metal regulatory elements affected expression of some of these systems. Finally heat inactivation may have denatured some plasma metal-chelating proteins, affecting expression of some of these systems.

While Jackson and Burrows¹¹² showed that a component of the Pgm⁺ phenotype was involved in Fe acquisition *in vivo*, Une and Brubaker¹¹³ demonstrated that a Pgm⁻ KIM strain was highly virulent via intravenous (iv) injection,¹¹³ indicating that the then unidentified Ybt system was not needed in septicemic plague or the latter stages of bubonic plague. We replicated this finding by retro-orbital (similar to iv) injection in the now genetically defined *apgm* mutation in strain KIM and demonstrated that strains with an *irp2* or a *psn* mutation are also highly virulent by this route of infection (Table 2).^{8, 55} However, the majority of mice survived doses between 10-20 cells of the *irp2* mutant while all mice given similar doses of the Ybt⁺ parent succumbed to disease.⁸ Thus Ybt may play a modest, but not essential, role in septicemic plague and in the systemic stages of infections from low infectious doses. Using infection by oriental rat fleas, Sebbane *et al.*¹¹⁸ showed that an *irp2* mutant was able to cause septicemic plague in mice at low incidence. Recently, Tn-Seq analysis by Palace *et al.* found that mutants with insertions in *psn*, *ybtP* or *ybtA* were selected against in a mouse model of septicaemic plague.¹¹⁹ This suggests that in mixed infections, Ybt⁺ strains out compete mutants unable to use Ybt (e.g., *psn*, *ybtP* or *ybtA* mutants). However, in the absence of Ybt⁺ competitors, the Ybt system is not required for septicemic plague or the latter stages of bubonic plague but is essential in the early dermal/lymphatic stages of bubonic plague (Table 2).

Relatively few other metal transporters have been tested in the mouse model of septicemic plague. A *Apgm ΔyfeAB yopJ psa* mutant was avirulent in our mouse model of septicemic plague (Table 2).²² This degree of virulence loss may be due to other components within the *pgm* locus (Fet-Flp or non-metal related factors) in combination with reduced Mn and/or Fe acquisition due to the *ΔyfeAB* mutation. In addition, background mutations in *yopJ* and *psa* in combination with the *ΔyfeAB* mutation may have increased virulence loss. However, the *yopJ* and *psa* mutations, designed to slightly attenuate this strain, did not cause a substantial loss of virulence in the absence of the *ΔyfeAB* mutation (Table 2).²²

As expected, a single *znu* mutant was fully virulent in this model. However the *irp2 znu* mutant had over a 4 x 10⁵-fold loss of virulence. The high virulence of a *psn znu* mutant demonstrates that the loss of virulence by the *irp2 znu* mutant was due to the defect in Zn acquisition and not a combination of defects in Zn and Fe acquisition (Table 2).⁸ While a large number of transition metal systems remain to be tested, the Yfe system for Fe and Mn transport as well as Znu and Ybt for Zn transport play a role in septicemic plague (Fig. 7B).

Pneumonic Plague

Secondary pneumonic plague is an occasional complication of bubonic or septicemic infection that occurs from hematogenous spread to the lungs. In humans and non-human primates (but not rodents) with secondary pneumonic plague transmission can occur by infected respiratory droplets causing primary

pneumonic plague. Symptoms of fever, cough, and chest pain develop rapidly and radiology reveals a patchy bronchopneumonia or confluent consolidation of the lungs. While bacteria spread hematogenously to other internal organs, pneumonia is typically the cause of death in untreated cases. Untreated, fatality rates approach 100% and treatment with antibiotics delayed more than 24 h after symptoms develop is often unsuccessful^{100, 108, 109, 120, 121}

A microarray analysis of *Y. pestis* cells isolated by bronchioalveolar lavage from the lungs of mice 48 h post intranasal infection was compared to cells grown in Brain Heart Infusion broth at 37°C. At this point in the infection mice are showing signs of illness, some lung necrosis, and bacteria appear to be extracellular in alveoli and the small bronchioles.^{116, 121} Compared to *in vitro* grown cells, *Y. pestis* cells from the lungs showed moderately increased expression of *ybt*, *yfe* and *fet-flp* but not other Fe transporters (Table 1).¹¹⁶ Moderately lower expression of *feoAB* in the lungs is curious, although *in vitro* these genes are not Fe repressed during aerobic growth. These results suggest a moderately iron-deficient environment with necrosis and immune responses possibly affecting iron-chelation mechanisms. Although *yfeA* exhibited moderate differential expression (2.8-fold), *mntH* levels were similar *in vivo* and *in vitro*. All three genes used as indicators for Zn levels show increased expression in the lung compared to *in vitro*. Thus the lung appears to be a Fe- and Zn-deficient environment with Mn levels less clear.

Analysis of Ybt system mutants in the mouse intranasal infection model yielded the intriguing result that mutations in *irp2* were more highly attenuated (790-fold loss) than those in *psn* (33-fold loss). Thus mutants able to synthesize but not transport Ybt are 24-fold more virulent than mutants unable to synthesize Ybt. Both mutants exhibited significantly increased time-to-death (TTD) compared to much lower doses of the parent Ybt⁺ strain. The LD₅₀ and time-to-death results are the opposite of the *in vitro* phenotype in which transport mutants have a more severe growth defect than biosynthesis mutants (Table 2).⁵⁵ There are a number of different possible mechanisms for this difference, some with supporting *in vitro* evidence: 1) an unidentified, Ybt-YbtA-regulated gene might be required for virulence via the respiratory route – this gene would be transcriptionally active in the *psn* mutant but not in an *irp2* mutant; 2) Ybt may have direct toxic effects in the lungs; 3) Ybt may affect host immune cell recruitment and/or cytokine signalling; 4) Cu-Ybt complexes may reduce Cu toxicity; 5) the SOD activity of Cu-Ybt may play a role in the lung; and 6) in the lungs, Ybt plays a greater role in Zn acquisition.^{8, 55, 66, 94} Note that Zn uptake via Ybt would not be affected by a *psn* mutation⁸. Clearly, the Ybt system is a critical component in inhalation disease (Fig. 7C) with differential effects due to biosynthetic versus transport mutants.

We also found that a Δ *pgm* mutant had an increased TTD and an LD₅₀ of >4 x 10⁶ in this model; that is a 15-fold greater loss of virulence than the *irp2* mutant (Table 2).⁵⁵ Lee-Lewis and Anderson¹²² found that the Δ *pgm* KIM strain had a delay in TTD and did not cause a typical pneumonic infection. While bacteria survived in the lungs, they did not proliferate but spread hematogenously to other internal organs with septicaemia, not pneumonia, as the likely cause of death. Thus, the increased TTD of our *irp2* and *psn* mutants⁵⁵ might indicate death due to septicaemia not pneumonia. Supplementation with iron administered intraperitoneally reduced survival time but did not restore pneumonic disease, suggesting that an additional virulence factor(s), unrelated to Fe-acquisition, is encoded

within the *pgm* locus.¹²² It should be noted that other researchers have determined LD₅₀s ranging from 10⁴ to 10⁶ with Δ *pgm* strains of KIM and CO92 in mice.^{114, 122, 123} In some cases, BALB/c mice, which are more sensitive to plague were used; however, the reason(s) for the large differences in LD₅₀s remains to be fully examined and explained.

Our analysis of *yfe feo* (Fe transport), *yfe mntH* (Mn transport) and *znu* mutants also demonstrated full virulence in the mouse model of pneumonic plague (Table 2). Analysis of a *yfe feo efe fet-flp* mutant is needed to determine whether or not ferrous transport processes are needed in lung infections. Analysis of a *znu ybtX* mutant should determine whether this more severely Zn-transport-deficient mutant will be attenuated. Nevertheless, the differences in virulence of the *yfe feo* and *yfe mntH* mutants in bubonic versus pneumonic infection models are intriguing.

Conclusions and future directions

Under *in vitro* aerobic conditions, the Fe-Ybt transport system is the most important of the multitude of Fe transporters. Under microaerobic conditions, the Yfe, Feo, and Fet-Flp systems each contribute to Fe-deficient growth. The Yfe and MntH Mn transporters have apparently redundant roles *in vitro* while the Znu system is primarily responsible for Zn acquisition. However, a second Zn transporter involving the Ybt siderophore and YbtX also functions *in vitro*.

Virulence studies with *Y. pestis* indicate that *in vitro* and *in vivo* phenotypes of metal transport mutants may differ significantly.^{10, 53} The Mn transport double mutant has a modest *in vitro* growth defect but a more severe virulence defect in the bubonic plague model.¹¹ In contrast, the *znu* mutant has the opposite phenotype. A *znu* mutant has a severe *in vitro* growth defect while a second *irp* mutation is required for virulence loss in the septicemic plague model.^{8, 60}

Our *in vivo* studies have also clearly demonstrated that different metal transporters are required in specific organs or disease stages. The most striking example is Ybt: the three forms of plague show differing requirements for the Ybt system with pulmonary infections also showing an intriguing difference between Ybt biosynthetic mutants versus transport mutants.⁵⁵ In addition, the combination of Yfe/Feo Fe transporters and Yfe/MntH Mn transporters are important for bubonic plague progression but not pneumonic plague.^{11, 26}

Except for Ybt, the Fe, Mn and Zn transporters that may be important in pneumonic disease have not been identified. For *Y. pestis* strains with functional Ysu and/or Ych systems, studies are clearly needed to determine the *in vivo* importance of these siderophore-dependent transport systems. In CO92 and two endemic strains, the functional Ysu-Ych system does not compensate for loss of Ybt *in vivo*. Further studies with the septicemic model are required to identify whether transporters are important in the early lymphatic stages of bubonic disease, the later septicemic stage, or both.

The demonstration that Ybt is involved in Zn acquisition *in vitro* and *in vivo* is an exciting new development. However, direct binding of Zn by Ybt has not been demonstrated and it is possible that the siderophore plays an indirect role. In addition, an OM receptor (if required) has not been identified for uptake via YbtX. Since YbtX is not involved in Fe-Ybt uptake, we can use the *znu ybtX* mutant to examine the role of Zn acquisition in bubonic and pneumonic plague.

While great progress has been made in our understanding of transition metal uptake systems in *Y. pestis* and their roles in causing disease, much remains to be elucidated.

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Dr. Alexander Bobrov (left) earned his M.D. in Pediatrics at the Saratov State Medical University in 1991 and a Ph.D. in Microbiology at the Microbe Anti-Plague Institute in 1995. He worked there for four years and was promoted to Senior Scientist. Alex started as a postdoctoral researcher at the University of Kentucky (UK) in 1999 and has continued as a Research Associate since 2002.

Dr. Jacqueline Fetherston earned a B.S. in medical technology at Michigan State University (MSU) in 1974 and obtained her Ph.D. in cell biology in 1981 at Washington University. She performed postdoctoral research at MSU and the National Institutes of Health, U.S.A. She was a Research Associate at UK and a Senior Research Associate at the Louisiana State University School of Medicine in Shreveport (LSUMC-S). In 1991 she started as a Research Assistant Professor and has been promoted to Research Professor at UK.

Dr. Robert Perry (right) obtained his B.A. in biology at Indiana University in 1972 and earned his M.S. (1975) and Ph.D. (1978) degrees in microbiology at MSU. He performed postdoctoral research at Washington University, MSU, and UK. He was an Assistant Professor of microbiology at LSUMC-S from 1986-1991. In 1991 he joined the Department of Microbiology and Immunology at the University of Kentucky where he was promoted to Professor.

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