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Roles of the Yfe and Feo transporters of *Yersinia pestis* in iron uptake and intracellular growth

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Abstract In *Yersinia pestis*, the Yfe and Feo systems likely function to transport ferrous iron. Both FeoA and FeoB are essential for iron acquisition activity while FeoC is not. Mutations in *yfe* and *feo* had an additive effect on microaerophilic growth under iron-chelating conditions. *Y. pestis* cells lacking the Ybt siderophore-dependent system, the Yfe or the Feo system grow normally in J774A.1 cells. However, a double *yfeAB feoB* mutant was no longer able to grow in this murine macrophage cell line. This growth defect likely resulted from iron and not manganese deprivation since a *yfeAB mntH* mutant grew normally in J774A.1 cells. These results suggest that the Yfe and Feo systems are somewhat redundant ferrous iron transporters capable of iron acquisition during intracellular growth of the plague bacterium.

Keywords Plague · Feo · Yfe · Iron transport · Intracellular growth

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Plague, iron, and intracellular growth

Yersinia pestis, the causative agent of bubonic, septicemic, and pneumonic plague, has long been know to survive and grow in vitro in unactivated macrophages but not in PMNs. The importance of this ability in vivo is unclear. Although in vivo growth appears to be extracellular in most studies, *Y. pestis* has mechanisms for surviving in phagocytic cells as well as invading and growing in non-phagocytic cells (Perry and Fetherston 1997; Cowan et al. 2000; Pujol and Bliska 2005; Pujol et al. 2005).

In a number of pathogens, iron acquisition is critical for intracellular growth (Cianciotto 2004; Payne and Mey 2004). For example, *Shigella flexneri* appears to use a combination of SitA, Feo and aerobactin systems for growth in Henle cells (Runyen-Janecky et al. 2003) *Y. pestis* encodes 12 potential or proven iron transport systems—two heme transporters, four siderophore-dependent systems, five ABC transporters, and an Feo transporter. The Has hemophore system appears to be non-functional while the aerobactin locus has a frameshift in *iucA* and aerobactin is not synthesized. The Yersiniabactin (Ybt) siderophore-dependent and Yfe systems have demonstrated roles in the pathogenesis of bubonic plague in mice. The Yfe system, which is homologous to the *Shigella* and *Salmonella* SitA systems, transports iron and manganese (Perry 2004;

Perry and Fetherston 2004). This article focuses on the *Y. pestis* Yfe and Feo transporters and their role in intracellular growth in vitro.

The Yfe ABC transporter

YfeA-D comprise a typical ABC transporter (Fig. 1) with a periplasmic binding protein (YfeA), a heterodimeric permease (YfeC and YfeD) and an ATP-binding protein (YfeB). A *yfeAB2031.1* deletion in a *Y. pestis* Ybt^- strain had a reduced ability to grow across an iron-chelator gradient plate. Deletion of *yfeE*, encoding a putative inner membrane protein of unknown function, delayed growth of the mutant on these gradient plates. The observed growth defects were alleviated by supplementation with iron but not with manganese or zinc and the Yfe system has been shown to transport iron and manganese. Fur transcriptionally regulates the *yfeA-D* promoter, but not the *yfeE* promoter, and responds to iron and manganese but not zinc. These studies suggest that the biological role of the Yfe system is the transport of ferrous iron and manganese (Perry 2004; Perry and Fetherston 2004).

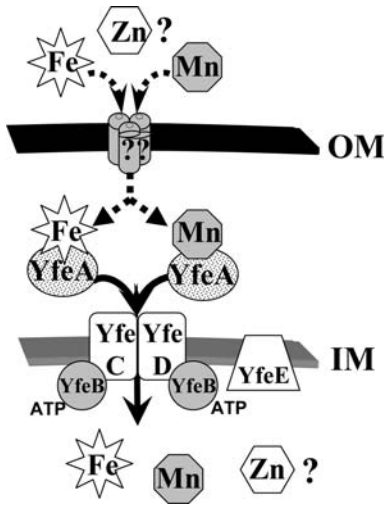

Fig. 1 Model of iron and manganese uptake via the *Y. pestis* Yfe ABC transporter. The channel through the outer membrane (OM) is unidentified as is the role for inner membrane (IM) protein YfeE. Whether zinc is transported by theYfe system remains to be determined

In a mouse model of bubonic plague, a Ybt^+ *yfeAB2031.1* strain in a slightly attenuated background ($YopJ^-$ Psa^-) had an ~70-fold loss of virulence compared to the Ybt^+ Yfe^+ parental strain. In a non-attenuated background, this loss of virulence is reduced. Ybt^- mutant strains are completely avirulent in the subcutaneous injection model of bubonic plague but fully virulent if injected intravenously, bypassing the initial lymphatic stage of plague. A double Ybt^- Yfe^- mutant was completely avirulent in this intravenous model suggesting that the Yfe system plays an important role in the latter stages of plague and that the Ybt system is essential in the early stages of bubonic plague but dispensable in the later stages (Perry 2004; Perry and Fetherston 2004).

The Feo transporter

Like other γ-proteobacteria, *Y. pestis* encodes a three gene *feoABC* locus (Fig. 2); *feoA* and *feoC* are small Orfs with unknown functions while FeoB is a putative permease with an *N*-terminal G-protein region and eight predicted transmembrane domains. Cartron et al. (Cartron et al. 2006) speculated that FeoA may interact with the G-protein domain of FeoB to maximally activate its translocase activity and that FeoC might be a transcriptional regulator of the putative *feoABC* operon. Although *feoA* and *feoB* are encoded in a large number of bacterial genomes, the transport function of this system has been studied in relatively few organisms. The Feo system has been shown to play a role in acquisition of ferrous

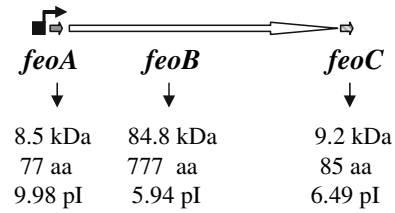

Fig. 2 Organization of the *Y. pestis feoABC* locus. Arrows indicate relative sizes of the three Orfs; the box and arrow represent the putative promoter region and transcriptional start. Predicted amino acid length, molecular mass, and pI of each *feo* Orf is shown

iron in *Escherichia coli, Helicobacter pylori, Legionella pneumophila, Leptospira biflexa, Shigella flexneri, Salmonella* species, and *Synechocystis* sp. PCC 6803. *Porphyromonas gingivalis* W83 has two FeoBs—one involved in ferrous iron uptake and the other in manganese uptake (Hantke 1987; Kammler et al. 1993; Tsolis et al. 1996; Velayudhan et al. 2000; Boyer et al. 2002; Runyen-Janecky et al. 2003; Dashper et al. 2005; Cartron et al. 2006).

Our research group has constructed nonpolar mutations in each of the three *feo* genes in *Y. pestis*. The mutants were analyzed for their ability to grow in candle jars on solidified, iron-deficient PMH medium containing a 0–3 mM nitrilotriacetic acid (NTA) gradients as well as under static growth conditions with liquid, iron-chelated PMH medium. The candle jar and static growth conditions generate a microaerophilic atmosphere. Even with microaerophilic growth conditions, the medium likely contained some ferric iron that could be used by the Ybt siderophore due to aerobic preparation of the medium and the lack of a reducing atmosphere during growth studies. In fact, Ybt^+ strains did not exhibit any growth defects under these conditions. Thus all studies were performed with Ybt^- strains. In a Ybt^- background, the Δ*feoB2088* mutant exhibited an ~50% loss of growth across the iron-chelator gradient by 40 h of incubation at 30°C; the growth pattern of this mutant and a *yfeAB2031.1* mutant are essentially identical (Fig. 3). The growth defect of the $FeoB^-$ mutant was complemented by expression of *feoB* in trans (unpublished observations). A Yfe^- $FeoB^-$ mutant showed a further growth defect compared to its Yfe^+ $FeoB^+$ parent, growing only across ~1/3 of the gradient plate (Fig. 3). No growth defects were observed under aerated conditions with any of the mutants. This suggests that the Feo and Yfe systems transport ferrous iron under the conditions we employed.

The *feoC::cam* mutant failed to show an iron-chelated growth defect in either a Ybt^- or a Ybt^- Yfe^- background (unpublished observations). However, a *feoA::kan* mutant in at Ybt^- background had a growth defect similar to that of the Ybt^- Δ*feoB* mutant in liquid PMH chelated with 10 μM ferrozine (Fig. 4). This growth defect was corrected by complementation with the cloned *feoA* gene indicating that the phenotype was not due to polar effects on *feoB* (unpublished observations).

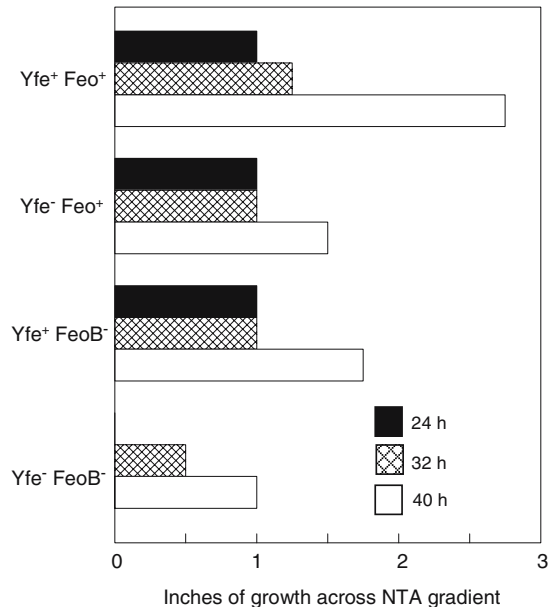

Fig. 3 Growth of *Y. pestis* strains at 30°C across PMH gradient plates containing NTA at 0–3 mM. Bars represent incremental growth against the concentration gradient at different times measured in inches from 0 (no growth) to 3 (confluent growth across the plate). Data shown are from one of two independent experiments. Strains: Yfe^+ Feo^+ - KIM6 (Δ*pgm* [Ybt^-] Yfe^+ Feo^+); Yfe^- Feo^+ - KIM6–2031.1 (Δ*pgm* [Ybt^-] Δ*yfeAB2031.1* Feo^+); Yfe^+ $FeoB^-$ - KIM6–2088 (Δ*pgm* [Ybt^-] Yfe^+ Δ*feoB2088*); Yfe^- $FeoB^-$ - KIM6–2031.1 (Δ*pgm* [Ybt^-] Δ*yfeAB2031.1* Δ*feoB2088*). The *pgm* locus is a 102 kb region of the chromosome that spontaneously deletes. The Ybt siderophore-dependent iron transport system is encoded in this region. Consequently, Δ*pgm* mutants are Ybt^-

Growth in J774A.1 cells

We have examined the effect of mutations in various iron transport systems on the ability of *Y. pestis* to grow in the murine macrophage cell line, J774A.1. Bacteria were grown in iron-deficient PMH medium at 37°C, prior to infection of a nearly confluent J774A.1 monolayer at an MOI of 10. Compared to the parental strain, mutations which eliminated the synthesis of the siderophore, Ybt, or its uptake had no effect on the growth of

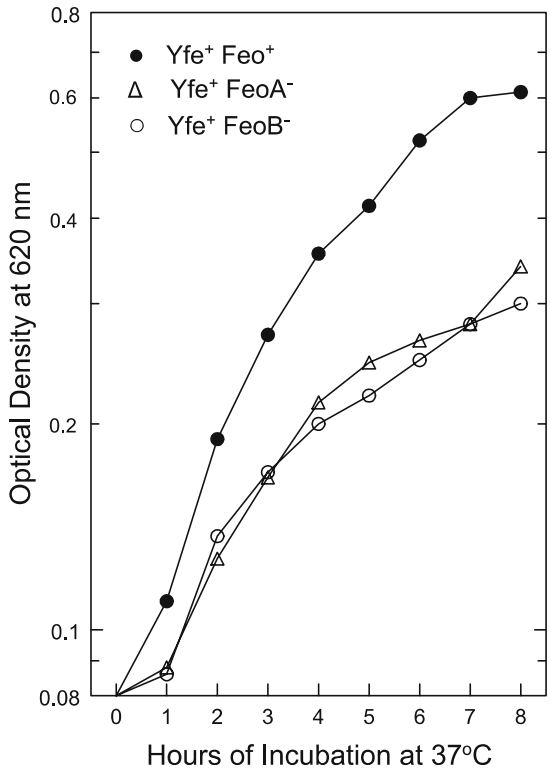

Fig. 4 Static growth of *Y. pestis* strains at 37°C in iron-deficient PMH containing 10 μM ferrozine. Strains: Yfe^+ Feo^+ - KIM6 (Δ*pgm* [Ybt^-] Yfe^+ Feo^+); Yfe^+ $FeoA^-$ - KIM6–2120 (Δ*pgm* [Ybt^-] Yfe^+ *feoA::kan2120*); Yfe^+ $FeoB^-$ - KIM6–2088 (Δ*pgm* [Ybt^-] Yfe^+ Δ*feoB2088*)

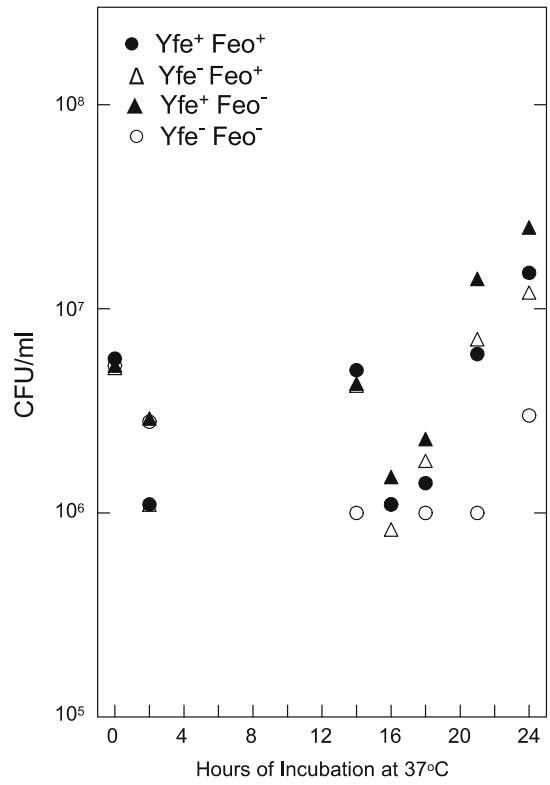

Fig. 5 Growth of *Y. pestis* strains at 37°C in cells of the murine macrophage cell line, J774A.1. Bacterial cells were grown in iron-deficient PMH at 37°C prior to infection of a nearly confluent J774A.1 monolayer at an MOI of 10. After 1 h of incubation, gentamicin (2 μg/ml) is added to kill extracellular bacteria (1 h incubation) and bacterial colony forming units (CFU) were determined by plating serial dilutions. Gentamicin was added to the infected monolayer 1 h prior to each sampling time

Y. pestis in J774A.1 cells. Similarly, mutants that lacked the Yfe ABC transporter or both the Yfe and Ybt systems were unaffected in intracellular growth in vitro (unpublished observations). Consequently, we examined whether the Feo ferrous uptake system played a role in intracellular growth.

An $FeoB^-$ mutant showed an intracellular growth pattern similar to the parental Ybt^- strain. For these strains, there is an initial killing phase (6–7 h) followed by bacterial growth such that by 24 h post-infection the intracellular bacterial population exceeded in initial input (Fig. 5). However, a $FeoB^-$ Yfe^- double mutant in a Ybt^- background displayed a severe intracellular growth defect failing to recover significantly after the initial killing phase (Fig. 5). Thus the plague Feo and Yfe transporters appear to have somewhat redundant functions that play an important role in intracellular growth.

Since the Yfe system transports manganese as well as iron, we constructed $MntH^-$ and $MntH^-$ Yfe^- mutant strains to determine if the growth defects were due to decreased manganese transport (Hazlett et al. 2003). MntH is a major manganese transport system in several bacterial species. Analysis of the plague genome did not identify any putative manganese transporters in addition to the Yfe and MntH systems. In contrast to the results observed with other pathogens, the *Y. pestis* manganese transport mutants displayed no intracellular growth defect in J774A.1 cells (unpublished observations). This suggests that the intracellular growth defect we observed in the Yfe^- $FeoB^-$ mutant was due to loss of iron uptake rather than manganese uptake.

Summary

In *Y. pestis*, the Yfe and Feo systems likely function to transport ferrous iron. Both FeoA and FeoB are essential for iron acquisition activity while FeoC is not. Mutations in *yfe* and *feo* had an additive effect on microaerophilic growth under iron-chelating conditions. *Y. pestis* cells lacking the Ybt siderophore-dependent system, the Yfe or the Feo system grow normally in J774A.1 cells. However, a double *yfeAB feoB* mutant was no longer able to grow in this murine macrophage cell line. This growth defect likely resulted from iron and not manganese deprivation since a *yfeAB mntH* mutant grew normally in J774A.1 cells. These results suggest that the Yfe and Feo systems are somewhat redundant ferrous iron transporters capable of iron acquisition during intracellular growth of the plague bacterium.

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