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Yersiniabactin iron uptake: mechanisms and role in *Yersinia pestis* pathogenesis

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Abstract

Yersiniabactin (Ybt) is a siderophore-dependent iron uptake system encoded on a pathogenicity island that is widespread among pathogenic bacteria including the Yersiniae. While biosynthesis of the siderophore has been elucidated, the secretion mechanism and a few components of the uptake/utilization pathway are unidentified. *ybt* genes are transcriptionally repressed by Fur but activated by YbtA, likely in combination with the siderophore itself. The Ybt system is essential for the ability of *Y. pestis* to cause bubonic plague and important in pneumonic plague as well. However, the ability to cause fatal septicemic plague is independent of Ybt.

Keywords

Yersiniabactin; *Yersinia pestis*; iron transport; siderophore; plague

1. Yersinia pestis and plague

Within the *Enterobacteriaceae* family, the genus *Yersinia* is composed of environmental species, a fish pathogen, two enteropathogenic species (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) and the plague bacillus *Yersinia pestis* which has caused three pandemics. However, plague is primarily a disease of rodents and their associated fleas. In nature, fleas feeding on a septicemic mammal become infected and the bacteria grow in the mid-gut of the flea. The infected flea transmits *Y. pestis* to mammals during subsequent blood meals via an early phase (blockage/biofilm-independent) mechanism and/or by a blockage/biofilm-dependent mechanism. From the bite wound, *Y. pestis* spreads via the lymphatics system to a regional lymph node and multiplies to high numbers causing necrosis and architecture destruction. This results in a swollen lymph node or bubo from which the name bubonic plague is derived. Bacteria spread through the bloodstream to the liver and spleen where they multiply and initiate a septicemia. High numbers of bacteria in the bloodstream are needed to ensure infection of a naïve flea feeding on the infected mammal. Untreated, fatality rates from bubonic plague can reach 50%. A small proportion of mammals develop septicemia with no bubo formation. This primary septicemic plague bypasses the lymphatic stage of the bubonic disease [1–4]. Although epidemic strains of *Y.*

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pestis (biovars Antiqua, Mediaevalis, and Orientalis) cause disease in animals and humans, enzootic strains cause disease in rodents but not humans [5–6].

During the course of bubonic plague, the lungs can become infected causing secondary pneumonic plague. Humans and non-human primates can then spread the disease via respiratory droplets causing primary pneumonic plague that is rapidly and nearly 100% fatal without treatment [2, 4].

2. Early studies leading to the discovery of the siderophore-dependent yersiniabactin (Ybt) iron transport system

A number of virulence determinants are important for the pathogenesis of bubonic, septicemic, and/or pneumonic plague. Some early observations noted as being important for the development of bubonic plague are now known to be due to the siderophore-dependent Ybt iron transport system. In 1956 Jackson and Burrows observed that spontaneous *Y. pestis* nonpigmented mutants (Pgm^-; unable to bind hemin at room temperature) were avirulent in mice via subcutaneous injection unless supplemented with iron or hemin [7]. Although beyond the genetic analysis of that day, this mutant was likely a spontaneous deletion of the 102-kb pigmentation (*pgm*) locus that includes the High Pathogenicity Island (HPI) that encodes genes of the Ybt system (see section 5.1 below). Almost 20 years passed before Wake *et al* proposed that *Y. pestis* produced siderophores (which they called siderochromes). Pgm^+ populations of *Y. pestis* had more siderophore producers than Pgm^- populations and the secreted siderophore inhibited the activity of the bacteriocin pesticin [8]. Only Pgm^+ cells of *Y. pestis* are sensitive to pesticin, a bacteriocin produced by *Y. pestis* whose activity is repressed by growth with iron or hemin [9–10]. It was subsequently shown that the outer membrane (OM) receptor for pesticin (designated Psn in *Y. pestis* or FyuA in *Y. enterocolitica*) mapped to the *pgm* locus in *Y. pestis* and serves as the receptor for the Ybt siderophore [11–13].

In 1987, Heesemann *et al.* identified a siderophore-like activity in *Y. enterocolitica* and *Y. pseudotuberculosis* which was later named yersiniabactin [14–15]. Haag *et al.* purified Ybt from *Y. enterocolitica* and showed that it was taken up through the pesticin receptor, FyuA [16]. Two different groups determined the structure of Ybt from *Y. enterocolitica* [17–18]. Perry *et al* later confirmed that Ybt from *Y. pestis* had the same structure [19] (Fig. 1). In 1987, Carniel *et al.* identified two iron-regulated proteins (HMWP1 and HMWP2 encoded by *irp1* and *irp2*, respectively) that were expressed only in high virulence or pathogenic Yersiniae strains and determined that a portion of the *irp2* gene was absent in low virulence or nonpathogenic strains [20–21]. HMWP1 and HMWP2 are required for Ybt biosynthesis [22]. Thus by the end of 1990, the Ybt structure and some components involved in its biosynthesis and uptake had been identified.

3. Biosynthesis of Ybt

Ybt is a member of a siderophore class whose Fe^{3+}-binding groups include thiazoline, oxazoline, or methyloxazoline rings. Its synthesis proceeds by a mixed nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) mechanism (Fig. 1). The siderophore is assembled from salicylate, three cysteines, a malonyl linker group and three methyl groups donated by S-adenosylmethionine to yield a four-ring structure composed of salicylate, one thiazolidine, and two thiazoline rings with a malonyl linker separating the final thiazoline from the thiazolidine ring (Fig. 1) [17–19, 23–24]. The requirement for seven gene products (HMWP1, HMWP2, YbtD, YbtE, YbtS, YbtT, and YbtU) for in vivo Ybt synthesis has been clearly demonstrated. Activated Ybt components are tethered to HMWP1 and HMWP2 by the 4′-phosphopantetheine moiety of coenzyme A which is added

to sites in the carrier protein domains of these enzymes by the YbtD phosphopantetheinyl (P-pant) transferase. YbtS synthesizes salicylate from chorismate while YbtE adenylates salicylate and transfers this activated compound to HMWP2. HMWP1 and HMWP2 serve as a scaffold for the synthesis of the siderophore (Fig. 1). NRPS enzymatic domains in HMWP2 cyclize and condense two cysteines to form two thiazoline rings linked to the salicylate moiety. The first four domains of HMWP1 are a PKS module that bis-methylates and reduces a malonyl linker moiety. Two NRPS domains of HMWP1 cyclize and condense the third cysteine molecule to form the final thiazoline ring. YbtU reduces the middle thiazoline ring to thiazolidine and the terminal thioesterase (TE) domain of HMWP1 releases the completed siderophore (Fig. 1). The YbtT putative type II thioesterase likely removes aberrant molecules from the enzyme complex (not depicted in Fig. 1) [22–30].

In *Y. pestis*, a *ybtT* mutation reduces Ybt synthesis by ~75% compared to the Ybt^+ parent strain. An alanine substitution for S or H in the putative thioesterase catalytic triad (S94-D172-H230) of YbtT yielded a phenotype similar to a *ybtT* deletion. In addition, reconstitution of the *Y. pestis* Ybt system in *E. coli* without YbtT resulted in 2.5-fold less Ybt than reconstitution with YbtT. However, *in vitro*, Ybt synthesis is efficient without YbtT and addition of the purified protein did not increase Ybt synthesis. This is likely because only correct precursor molecules were provided in the *in vitro* reaction mix, making mischarging of molecules onto HMWP1 and HMWP2 unlikely [27, 30–32]. Taken together these results support the contention that YbtT is an editing thioesterase that removes misprimed or aberrant intermediates from the Ybt synthetase complex. It also suggests that *in vivo* Ybt synthesis is relatively error prone.

The formation constant of Ybt with ferric iron is 4×10^{36} and the proposed iron binding sites are identified by asterisks in Fig. 1. The middle thiazolidine ring may provide needed flexibility in the molecule that allows binding of iron with high affinity in a 1:1 ratio. The crystal structure of the Fe-Ybt complex has been solved and confirms that iron is bound by the three nitrogen electron pairs and three negatively charged oxygen atoms depicted in Fig. 1 [19, 33].

4. Ybt transport

4.1 Export of Ybt

After synthesis of the siderophore is completed, Ybt is secreted from the bacterial cell by an undetermined mechanism. YbtX, an inner membrane (IM) protein with 12 predicted transmembrane domains, is tentatively assigned as part of the Ybt efflux system (Fig. 2) due to modest similarities to EntS and AlcS, exporters of enterobactin and alcaligin siderophores in *E. coli* and *Bordetella*, respectively [34–35]. However, YbtX also has similarities to RhtX and FptX which import the siderophores rhizobactin and pyochelin in *Sinorhizobium meliloti* and *P. aeruginosa*, respectively [36–37]. Experimentally, a *Y. pestis ybtX* mutant is not defective in Ybt secretion, uptake of iron from Ybt, or growth under iron-restrictive conditions [38]. Therefore the function of YbtX remains elusive. If it is involved in Ybt secretion, it is not essential to that process.

4.2 Uptake of iron from Ybt

Iron uptake from Ybt is better, but not completely, characterized (Fig. 2). The OM protein Psn (termed FyuA in *Y. enterocolitica*) is required for sensitivity to the bacteriocin pesticin and iron uptake via Ybt. Psn functions have been shown to be TonB-dependent. Although not proven in *Y. pestis*, in other Gram-negative bacteria TonB requires ExbB and ExbD for its function. Homologues of these genes appear to form an operon distal from *tonB* in *Y. pestis. Y. pestis* strains with mutations in either *psn* or *tonB* exhibit similar defects in iron-deficient growth and iron uptake from submicromolar levels. Although *Y. pestis* has a

second TonB-like gene (*hasB*), HasB cannot substitute for TonB in iron uptake via the Ybt system [11, 13, 39–41].

In a deferrated, defined medium, a *Y. pestis psn* mutant shows greater defects in growth (Fig. 3) and uptake of iron when compared to a *Y. pestis irp2* mutant. The *psn* mutant makes and secretes Ybt siderophore but it is unable to use it while the *irp2* mutant cannot synthesize the siderophore [19, 42]. Secreted Ybt likely binds residual iron in the medium (~0.3 μM) making it unavailable for uptake by other functional *Y. pestis* iron uptake systems causing transport mutants to be more defective in iron acquisition than Ybt biosynthetic mutants. This is supported by the observation that a *psn irp2* double mutant has a growth phenotype similar to the *irp2* mutant (Fig. 3). Growth of this double mutant is inhibited by the addition of exogenous Ybt [42].

After passage through the OM into the periplasm is accomplished, the YbtP-YbtQ ABC transporter is required for uptake of Ybt-bound iron into the bacterial cell. YbtP and YbtQ are similar IM proteins with both permease and ATPase domains [38]. These fused-function types of ABC transporters are usually components of Type I secretion systems [43]. Nevertheless, strains with mutations in *ybtP* or *ybtQ* have an in vitro phenotype similar to a *psn* mutant – reduced growth and iron uptake without a significant reduction in Ybt siderophore secretion [38]. It is unclear whether a periplasmic binding protein (PBP) is required for this uptake system. No identifiable candidate lies within the HPI (see section 5.1) that encodes nearly all other *ybt* genes [23, 44]. However, studies with the *Y. enterocolitica* Ybt system suggest that additional component(s) are required. Brem *et al*. demonstrated that a *Y. enterocolitica* serotype O:5, biogroup IA strain (which does not encode the Ybt system) is unable to use Fe-Ybt even when expressing Psn, YbtP and YbtQ (FyuA, Irp6, and Irp7, respectively, in *Y. enterocolitica*) while a similarly transformed *E. coli* strain lacking the Ybt pathogenicity island was able to use Fe-Ybt. They speculated that O:5, biogroup IA strain either lacks the PBP essential for uptake or was unable to remove iron from the siderophore [45].

Since transport studies have used radiolabeled iron [19, 38], uptake of a Fe-Ybt complex into the bacterial cell has not been demonstrated. It remains a possibility that iron could be removed at the cell surface, in the periplasm, or after transport into the cell cytoplasm. While Fig. 2 shows transport of the Fe-Ybt complex into the cytoplasm and release of iron there, the actual mechanism and site of iron release from Ybt remains to be characterized.

For other siderophores, iron release is achieved either by reduction to ferrous iron and release due to the low affinity of most siderophores for ferrous iron or by degradation of the siderophore to release the bound ferric iron. Genes encoding either type of mechanism do not lie within the HPI that encodes most other *ybt* genes.

5. Genetics and transcriptional regulation of the Ybt system

5.1 The *ybt* locus and high pathogenicity island (HPI)

The *ybt* loci in *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* have been sequenced and found to be 97–100% identical. The locus consists of 4 operons; two are monocystronic, one contains 5 genes that encode biosynthetic enzymes, while the fourth operon has genes encoding proteins involved in Ybt uptake, biosynthesis, and possibly export (Fig. 4). Except for *ybtD*, *fur*, and additional transport components (i.e., *tonB*, *exbB*, and *exbC*) all other genes shown to be involved in the function or regulation of the Ybt system are encoded within this locus [23, 25, 46].

The *ybt* locus lies within the HPI which was first identified in the three mammalian-pathogenic species of *Yersinia.* In turn, the HPI of *Y. pestis* lies within and comprises approximately one third of the *pgm* locus. The HPIs in *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* vary in size, associated insertion sequences, genomic insertion sites, and nucleotide sequences outside of the *ybt* locus in the "left-hand" portion of the HPI [46]. Excision and horizontal transfer of the HPI has been demonstrated in *Y. pseudotuberculosis* [47–48].

The HPI is widely distributed among the members of the *Enterobacteriaceae* family being found in species of *Citrobacter, Enterobacter, Klebsiella, Photorhabdus, Salmonella, Serratia,* and all of the *E. coli* pathotypes [46]. The IS*100* associated with the *Y. pestis* HPI is absent in the other *Enterobacteriaceae*. Recently, the *ybt* locus has been detected in *Pseudomonas syringae* and found to be functional in 10 of 15 pathovars of this organism. However, this *ybt* locus may have been acquired prior to insertion into the HPI [49]. Most of the organisms that possess the HPI and have been tested produce the HMWP1 and HMWP2 proteins in an iron-repressible manner and synthesize the Ybt siderophore [46].

5.2 Fur regulation

Y. pestis produces a typical Gram-negative Fur [Iron (Fe) Uptake Regulation] protein that represses the transcription of promoters with a Fur binding sequence (FBS or Fur box) when cells are grown with excess iron [50–52]. Each of the four *ybt* operons (Fig. 4) within the HPI have promoters with FBSs (Fig. 5A and 5B) and are repressed through Fur approximately 12-fold (*psn*), 8-fold (*irp2-irp1-ybtUTE*), 11-fold (*ybtA*) and 55-fold (*ybtPQXS*) during growth with 10 μM iron compared to growth in deferrated, defined media (PMH/PMH2) without additional iron [38, 53–54]. In contrast, *ybtD,* which is encoded outside of the HPI and *pgm* locus, is not regulated by Fur, iron, YbtA or the Ybt siderophore. The lack of regulation by Fur or YbtA suggests that YbtD may have been co-opted for use as the P-pant transferase that allows attachment of activated substrates to HMWP1 and HMWP2 [25]. Using the enzootic 201 strain, Gao *et al* demonstrated high-affinity binding of purified *Y. pestis* recombinant Fur-Mn (routinely used for in vitro analyses) to the four *ybt* locus promoter regions. Fig. 5A shows the FBSs derived from these studies [50].

5.3 Transcriptional regulation by YbtA and the Ybt siderophore

The earliest indication that the Ybt siderophore served as a signal to activate transcription of *ybt* genes was the observation that an *irp2* mutant had a lower level of Psn protein. Normal Psn levels were restored by adding supernatant containing Ybt but not supernatant lacking the siderophore to an *irp2* mutant culture [11]. Subsequent studies used transcriptional reporters to demonstrate a role for the siderophore in regulation. Compared to a Ybt-producing strain, an *irp2::kan* mutant had 17-, 2-, and 22-fold losses in transcription from the *irp2, psn,* and *ybtP* promoters respectively [38, 53–54]. Time course studies revealed that transcription from a *ybtP::lacZ* reporter was increased 4-fold 10 min after Ybt addition. Furthermore, activation was caused by adding Ybt at a concentration 500-fold lower than that required to stimulate growth of a biosynthetic mutant. Thus Ybt is a potent signaling molecule [54]

Ybt likely acts in concert with YbtA, a transcriptional regulator in the AraC family. YbtA is a negative regulator of its own expression and a transcriptional activator of the *irp2, psn,* and *ybtP* promoters (Fig. 4 and 5B). Under iron-deficient conditions, a *ybtA::kan* mutation caused a 50-fold drop in transcription from the *psn* promoter compared to the parent strain. Similar studies showed 109- and 16-fold losses in activation of the *ybtP* and *irp2* promoters,

respectively for the *ybtA::kan* mutant compared to its parent strain. In contrast, this same mutation caused a 2.8-fold increase in transcription from the *ybtA* promoter [38, 53–54].

Comprehensive analysis of the effect of mutations in 11 of the 12 known *ybt* genes (Fig. 4) and *tonB* on transcription from the *ybtP* promoter revealed a more complex story. The *ybtA* mutant had the lowest level of *ybtP* expression while mutations in genes required for uptake (*tonB, psn,* and *ybtP*) had modestly increased transcription (1.7-fold) compared to the Ybt^+ strain. As described earlier (Section 4.2), these mutants are likely more iron starved due to siderophore secretion without subsequent iron acquisition. Mutations in *ybtD, ybtE, ybtU* and *irp1* caused a loss of expression similar to that of the *irp2* mutation (~18-fold) while a mutation in *ybtX* had no effect on transcription. In contrast, mutations in the thioesterase domain of HMWP1 (*irp1-2086*), *ybtS*, or *YbtT* caused a slight increase in *ybtP* expression (1.4-fold). These mutants, like the other Ybt biosynthetic mutants, failed to produce Ybt by a growth-stimulation bioassay yet they have a very different regulatory phenotype. Subsequent HPLC analysis demonstrated that the *ybtT* and *irp1-2086* mutants produced 23% and 3%, respectively, of authentic Ybt compared to their Ybt^+ parent. These lower levels of Ybt are apparently sufficient for regulatory signaling but insufficient for providing iron for growth. The *ybtS* mutant produced no detectable authentic Ybt. However it is possible that this mutant makes a “Ybt-like” molecule with another phenolic group substituted for salicylic acid. While this, and a possible second “Ybt-like” molecule, are biologically irrelevant for normal regulation, their identification and structural comparison with Ybt will help identify key chemical structures necessary for transcriptional activation. [31, 54].

It is puzzling that mutations in genes involved in Ybt uptake (*ybtP, psn, tonB*) have no apparent defect in transcriptional activation. We hypothesized that the Fe-Ybt complex enters the cytoplasm and interacts with YbtA to activate transcription from the *irp2, psn,* and *ybtP* promoters (Fig. 5B). In some systems, binding of the external siderophore to the siderophore receptor transmits a signal through the membranes. However, in that case, mutations in the OM receptor or TonB prevent signal transduction [55–56]. In *Y. pestis*, mutations in *tonB* or *psn* slightly increase transcription rather than causing a loss of transcriptional activation [54]. In these mutants, sufficient Fe-Ybt might enter through alternate, non-specific transport components. Alternatively, increased Ybt production by uptake mutants could saturate the secretion system artificially allowing unsecreted, cytoplasmic Ybt to activate transcription.

The promoter regions of *psn* (*fyuA* in *Y. enterocolitica*), *irp2,* and the intergenic region of the divergent promoters for *ybtA* and *ybtP* (*irp6* in *Y. enterocolitica*) have similar inverted repeat sequences (RSs) with the promoter-proximal RS partially overlapping the −35 region for *ybtP, psn,* and *irp2* (Fig. 5C) [13, 28, 53]. Mutation of the first 9 bp to disrupt the promoter-distal RS of *psn* (Fig. 5C) caused a 4-fold loss of transcription compared to the parent promoter. This suggested that the RSs are required for full expression possibly through binding of YbtA [53]. Anisimov *et al* demonstrated that purified YbtA binds to the promoter regions of *Y. enterocolitica irp2* and *fyuA* and protects an ~50 bp region that includes both RSs (RS1 and RS2). Addition of either deferrated Ybt or Fe-Ybt did not affect mobility shifts or the protected promoter regions. Binding of YbtA to the *Y. pestis* and *Y. enterocolitica ybtA-irp6/ybtP* intergenic region differs from binding to the *irp2* and *fyuA/psn* promoters in that there were two shifted bands and the protected region was twice as large, including both transcription starts, −10, −35, and FBSs. This extended protection lead Anisimov *et al* to suggest a third RS (RS3; Fig. 5C) although the sequence similarity to other RSs is modest. Deletion of RS2 or RS3 eliminated the upper shifted band and an ~2-fold increase in transcription from *irp6/ybtP*. Deletion of RS1 alone resulted in almost no binding of YbtA and loss of YbtA inhibition of *ybtA* transcription [57–58]. Overall, these

results clearly show that YbtA binds to the *ybt* promoter regions and that this interaction involves the RS sequences.

The *Y. enterocolitica ybtA/irp6* intergenic promoter region contains an ERIC sequence not found in *Y. pestis* or *Y. pseudotuberculosis*. This sequence does not affect the RS consensus sequence or the overall regulation of the *Y. enterocolitica ybt* genes. However a *Y. enterocolitica ybtA* promoter fusion had significantly higher transcriptional activity than the analogous *Y. pestis ybtA* reporter [11, 22, 38, 59].

6. Iron use and plague pathogenesis

6.1 Iron sources used by Ybt

Similar to other siderophores, Ybt has no affinity for ferrous iron. Its affinity for ferric iron ($\sim 4 \times 10^{36}$) is higher than a number of other siderophores and indicates that Ybt should be able to remove iron from a variety of host iron-binding proteins [19]. A Ybt^+ strain but not a Ybt^- mutant will grow in the presence of partially iron-saturated transferrin or lactoferrin even when the cells are separated from these iron sources by a dialysis membrane which prevents contact between cell surfaces and transferrin or lactoferrin. Under these conditions, Ybt is necessary for the use of iron from transferrin and lactoferrin. The direct removal of iron from transferrin by Ybt was demonstrated using urea-polyacrylamide gels. Transferrin mobility in these gels is altered by the removal of iron from transferrin. Thus, Ybt removes iron from transferrin as well as other mammalian iron-binding proteins for nutritional use by the bacterium [42].

6.2 Bubonic plague and Ybt

Jackson and Burrows observation that spontaneous Pgm^- *Y. pestis* mutants (unable to bind hemin at room temperature) were avirulent in mice via subcutaneous injection unless supplemented with iron or hemin [7] implicated iron acquisition as important for the progression of plague. Later studies using defined *ybt* mutations demonstrated that the Ybt system is essential for bubonic plague. *Y. pestis* strains with mutations in biosynthetic or uptake genes in a mildly attenuated background had virulence losses of >660-fold by the subcutaneous route of infection. This degree of virulence loss is an underestimate since the highest bacterial doses tested were less than 10^5 cells [22, 38]. More recently, we have found that mutations in *psn* or *irp2* (in anotherwise wild-type background) result in subcutaneous LD_{50}s of $>10^7$ – a virulence loss of $>4 \times 10^6$ [42]. Thus loss of either Ybt uptake or Ybt synthesis results in a nearly complete inability to cause fatal bubonic disease [42].

6.3 Septicemic plague and Ybt

Despite being expressed in vitro in serum [60], studies suggest that the Ybt system is not required for septicemic plague. Une and Brubaker demonstrated that a Δ*pgm* mutant is fully virulent in mice via an intravenous route of infection [7, 61]. Since the *pgm* locus includes the *ybt* locus [23, 40, 62–63], this clearly eliminates a role for the Ybt system in septicemic plague. This conclusion was recently confirmed using a defined *irp2* mutation. In addition, using a flea-to-mouse infection model, Sebbane *et al* showed that the *irp2* mutant caused fatal plague in 20% of the mice compared to 90% for the Ybt^+ parent. Of the fatal *irp2* infections, histological examination of proximal lymph nodes indicated that one mouse had primary septicemic plague while the second had septicemic plague with a mild lympyhadenitis [64].

These results indicate that the Ybt system is critical only during the early lymphatics stage of bubonic plague. In a rat model of bubonic plague, *ybt* and other genes encoding iron

transport systems were highly expressed in bubos [65]. In *Y. enterocolitica* infections, *fyuA* (the gene encoding the Ybt receptor) and *hemR* (encoding an OM hemin receptor) were more highly expressed in the spleens of mice compared to the livers [66]. While this result indicates that the liver is less iron-restrictive than the spleen, overall these studies demonstrate that the Ybt system is expressed in liver and spleen. Consequently, other systems are likely more effective in acquiring iron once bacteria have disseminated via the bloodstream.

6.4 Pneumonic plague and Ybt

In contrast to bubonic plague where Ybt is essential and septicemic plague where Ybt is dispensable, in pneumonic plague, mutations in various components of the Ybt system have different effects on virulence. Two different mutations in *psn* had an LD_{50} of ~10^4 compared to the parent strain's LD_{50} of ~300 – an ~33-fold loss of virulence. In this infection model two different *irp2* mutations, causing loss of Ybt biosynthesis, had an LD_{50} of ~3×10^5 – approximately 790- and 24-fold virulence losses compared to the parent and *psn* mutants, respectively. Time-to-death analyses with infectious doses similar to the calculated LD_{50}s showed a two-day delay in the 50% endpoint for the *psn* mutant compared to the parent strain (4 vs 6 days) and a 4-day delay for the *irp2* mutant (4 vs 8 days) [42]. This suggests either that pneumonic disease progresses more slowly in *ybt* mutants or that mouse fatalities are due to systemic spread and septicemic disease. The latter possibility correlates with the findings of Lee-Lewis *et al* [67].

In contrast to infection with a Pgm^+ strain, Lee-Lewis *et al* found that mice infected intranasally with a *Δpgm* strain failed to show histological signs of pneumonia. In addition, iron supplementation of mice decreased the time-to-death from a high dose of *Δpgm* cells compared to Pgm^+ cells while the lungs still showed no histological signs of pneumonic disease. Instead mice appeared to die from septicemic plague [67]. Even with intraperitoneal injection of iron, BABL/c and C57BL/6 mice were more susceptible to the *Δpgm* strain than C3H mice. Both of the more susceptible mouse strains lack the natural resistance-associated macrophage protein 1 (NRAMP1) while the less susceptible C3H strain has this gene. NRAMP1 is involved in activating innate immune responses and killing intracellular bacteria, likely through the transport of iron, manganese, and zinc [67–68]

Our results and those of Lee-Lewis *et al* suggest not only that the *pgm* locus encodes additional virulence factor(s) important for pneumonic plague, but also that the Ybt siderophore may serve a virulence role in addition to iron acquisition [42, 67]. In vitro, growth defects under iron-deficient conditions are worse for mutants producing but unable to use Ybt compared to a mutant unable to produce the siderophore (Fig. 3). Secretion of the siderophore by the uptake mutant allows binding of residual iron (~0.3 μM) likely making it unavailable to the other *Y. pestis* iron transporters. Our *in vivo* results are the opposite of this *in vitro* phenotype. Thus we proposed that the Ybt siderophore also serves a non-Fe-acquisition role during lung infections [42]. We have suggested three possibilities: 1) Ybt may act as a signal molecule to transcriptionally activate unidentified, virulence factor genes in addition to *ybt* genes. 2) The Ybt molecule (possibly in conjunction with chelated iron) may have a direct toxic effect in the lung environment. Pyochelin is structurally similar to Ybt and has been shown to damage endothelial and epithelial cells by generating hydroxyl radicals. The siderophores desferrioxamine and enterobactin are cytotoxic for T cells in vitro. 3) Ybt affects immune cell function(s). Addition of purified Ybt has been shown to reduce the level of reactive oxygen species in J774A.1 cells, PMNs, and human monocytes *in vitro. In vitro* T-cell proliferation is inhibited by desferrithiocin, which has a structure similar to pyochelin and Ybt. Finally, a number of siderophores affect cytokine production in a variety of host cells *in vitro* [42]. These possibilities are not mutually exclusive. Thus, the non-Fe-acquisition role(s) of Ybt remain(s) to be elucidated.

7. Conclusions and future directions

Since the identification of the first Ybt protein over twenty years ago, great progress has been made identifying components of the biosynthetic and transport systems and in understanding how the system is regulated. Intriguing differences in the requirement for Ybt in bubonic, septicemic, and pneumonic plague have been uncovered. However a number of questions remain.

While the mechanisms involved in synthesis of Ybt have been largely elucidated, some aspects of Ybt transport, utilization and regulation remain obscure. Although YbtX appears to be involved in siderophore export, no essential component of the secretion apparatus has been identified. YbtP and YbtQ are essential for uptake of iron from Ybt, but it is unclear whether these fused-function permease-ATPases also require a PBP, a membrane spanning protein, or no other components. Whether the Fe-Ybt complex enters the periplasm and/or cytoplasm has not been experimentally determined. Indeed the mechanism used to release iron from the siderophore is unknown.

While the overall regulatory mechanisms have been identified, it remains to be determined whether or not YbtA binds the siderophore to affect transcription of *ybt* genes. Anisimov *et al* demonstrated binding of purified YbtA protein to promoter regions in the absence of siderophore suggesting that Ybt is not required for binding of the regulator to DNA [58]; however, the protein was purified from inclusion bodies and solubility issues may have affected its conformation. In *P. aeruginosa*, pyochelin (a siderophore structurally similar to Ybt) is required for binding of the related PchR transcriptional regulator to regulated promoters [69]. If YbtA does bind the siderophore, does it bind deferrated or Fe-Ybt? If the siderophore transmits a regulatory signal without binding YbtA, what are the intermediary components of this signaling pathway? In the absence of components required for uptake, how does regulatory signaling occur? Finally, in the case of a *ybtS* mutant, what is the nature of the molecule involved in activating transcription?

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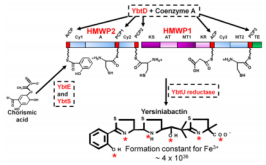


Fig. 1.

Biosynthesis of Yersiniabactin (Ybt). YbtD, a phosphopantetheinyl transferase, transfers the 4′-phosphopantetheine moiety of coenzyme A to carrier domains (red boxes) ArCP, PCP1, PCP2, ACP, and PCP3 which are attachment sites on HMWP1 and HMWP2 for the substrates adenylated salicylic acid, three cysteines, and malonate. Chorismic acid is converted to salicylic acid by YbtS. Salicylic acid is then adenylated by YbtE for attachment to the ArCP site of HMWP2. HMWP2 domains cyclize and condense two cysteines to form two thiazoline rings linked to the salicylate moiety while HMWP1 domains add the malonyl linker and convert a third cysteine to the final thiazoline ring. YbtU reduces the middle thiazolidine ring to a thiazoline ring to yield the final structure. Nonribosomal peptide synthetase (NRPS) domains are represented by blue boxes while polyketide synthase (PKS) domains are in purple. Aberrant or mischarged molecules on the enzyme complex are removed by YbtT, an editing thioesterase (not shown in diagram). The completed siderophore is released from the enzyme complex by the thioesterase (TE; green box) domain of HMWP1. The completed Ybt structure is shown with red asterisks identifying coordination sites for one Fe^{3+} atom. The disassociation constant (K_D) for Fe^{3+} is shown. HMWP1 and HMWP2 enzymatic domains: A, adenylation; ACP, acyl carrier protein; ArCP, aryl carrier protein; AT, acyltransferase; Cy, condensation/cyclization; KR, β-ketoreductase; KS, ketoacyl synthase; PCP, peptidyl carrier protein; MT, methyltransferase; TE, thioesterase. The diagram is reproduced with modifications from Miller *et al* [31] with the permission of the Society for General Microbiology.

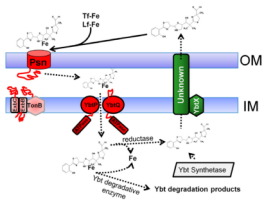


Fig. 2.

Ybt secretion and uptake. After release of the completed siderophore from the biosynthetic enzyme complex, Ybt is secreted from the cell by an unknown mechanism (green boxes). While there is evidence suggesting that YbtX is involved in secretion, it is not essential to this process. After release, Ybt can remove iron from transferrin (Tf-Fe) and lactoferrin (Lf-Fe). Although only uptake of radiolabeled iron has been experimentally demonstrated, our model favors uptake of the Fe-Ybt complex into the cell. The first step is binding to the TonB-dependent receptor Psn followed by translocation through the OM. Once in the periplasm Fe-Ybt is transported into the cytoplasm by YbtP/YbtQ. Whether additional components for this ABC transporter (e.g., a PBP or membrane spanning protein) are required for this step is undetermined. In the cytoplasm, Fe could be released from the siderophore by a reduction of Fe^{3+} to Fe^{2+} or by degradation of Ybt. Uptake components are in red with red lines denoting periplasmic and cytoplasmic domains. Dashed arrows indicate substrates or steps that are undetermined experimentally. The diagram is reproduced with modifications from Miller *et al* [31] with the permission of the Society for General Microbiology.

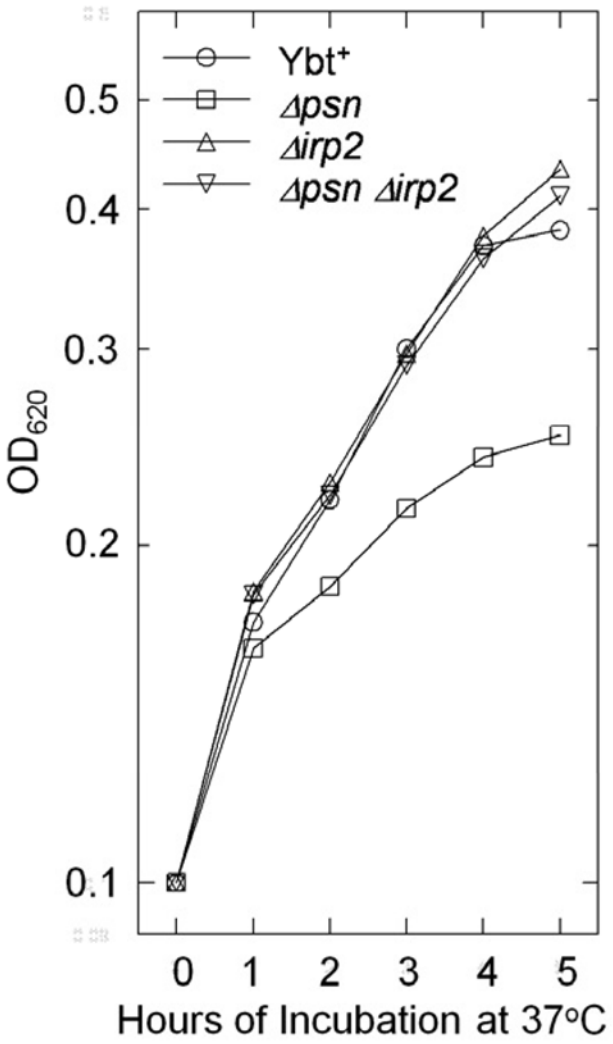
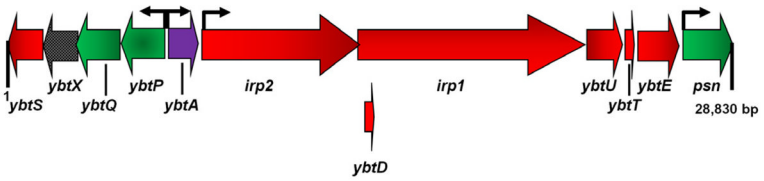


Fig. 3.

Growth of *Y. pestis* KIM strains in deferrated PMH2 at 37°C. A *Δpsn* mutant produces and secretes Ybt but cannot use the siderophore to obtain iron. A *Δirp2* mutant cannot make the Ybt siderophore. The *Δpsn Δirp2* mutant is defective in both biosynthesis and uptake. *Y. pestis* cells were acclimated to growth under iron-deficient conditions as previously described by Fetherston *et al* [42] which shows a similar experiment.



Protein (gene)	Predicted mass (kDa)	Function
YbtS	47.8	Ybt synthesis; Salicylate synthase
YbtX	44.7	unproven function; IM protein
YbtQ	66.5	Ybt uptake; IM permease-ATPase
YbtP	66.1	Ybt uptake; IM permease-ATPase
YbtA	35.7	Transcriptional regulator
HMWP2 (*irp2*)	228.5	Ybt synthesis; NRPS synthetase component
HMWP1 (*irp1*)	348.7	Ybt synthesis; NRPS/PKS synthetase component
YbtU	40.8	Ybt synthesis; thiazolinyl reductase
YbtT	29.1	Ybt synthesis; Editing thioesterase
YbtE	57.1	Ybt synthesis; Salicyl-AMP ligase
Psn	73.6/71.5	Ybt uptake; Ybt/pesticin OM receptor
YbtD	24.4	Ybt synthesis; P-pant transferase

Fig. 4.

Genetic organization of the *ybt* locus and *ybtD* in *Y. pestis.* The ~29 kb *ybt* locus encoding 11 genes in four operons (promoters are designated by small arrows) lies within the 102-kb *pgm* locus. The monocystronic *ybtD* gene is located outside of the *pgm* locus. Arrows designating genes indicate the direction of transcription/translation. Red arrows indicate a biosynthetic function, green arrows are for uptake components, and the black checkered arrow denotes the potential secretion component YbtX. The transcriptional regulator YbtA is represented by a purple arrow. Protein masses and functions are indicated in the chart below the genetic maps. The processed and unprocessed masses are given for Psn which has a signal sequence.

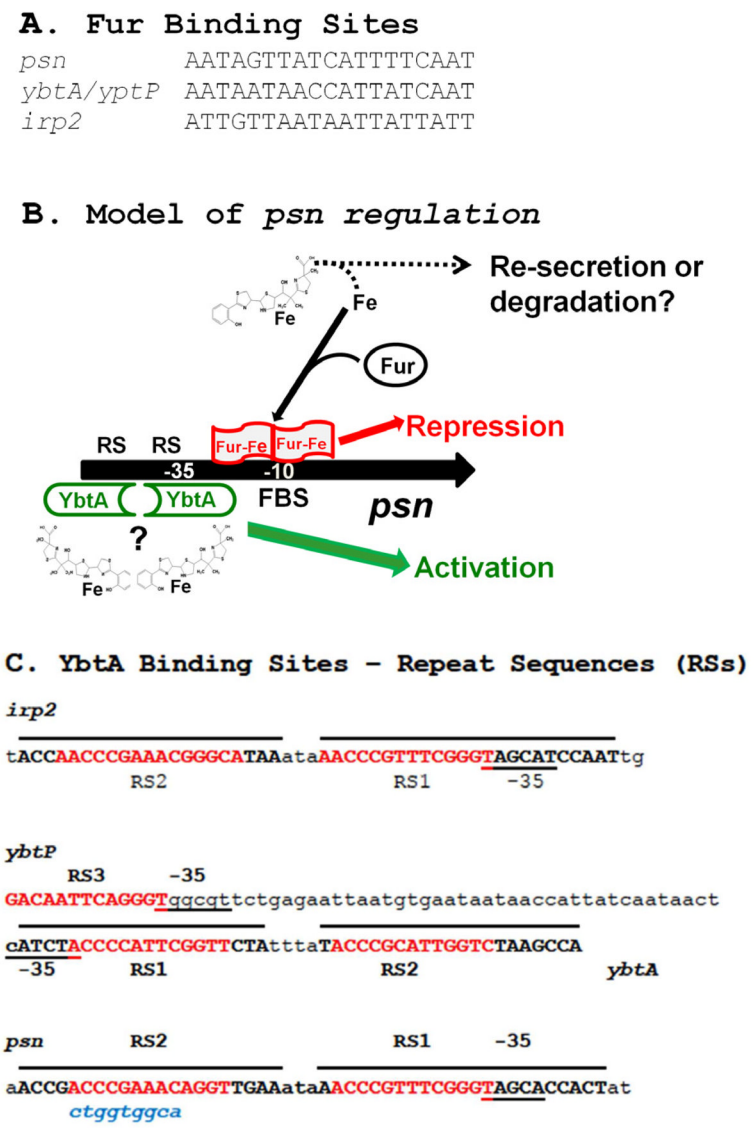


Fig. 5.

Transcriptional regulation by Fur and YbtA. (A) Fur binding sites (FBSs) shown were mapped by Gao *et al* [50] and the sequences shown are from *Y. pestis* strain 201. The *ybtP* FBS is part of the *ybtP/ybtA* intergenic region and identical to the *ybtA* FBS. (B) Model of *psn* regulation. During iron-sufficient conditions, the *psn* promoter is repressed through Fur-Fe binding to an FBS that overlaps the −10 region. Under iron-deficient conditions this repression is relieved due to a shift to primarily iron-free Fur. If Ybt siderophore is present, YbtA activates transcription of this promoter by binding to repeat sequences (RSs), one of which overlaps the −35 region. The *irp2* and *ybtP* promoters are regulated in the same manner. In contrast, YbtA represses transcription of its own promoter (not shown). Question marks indicate unresolved questions in our model. Our regulatory model favors a mechanism in which Fe-Ybt binds to YbtA for regulatory activity. However, binding of Fe-Ybt or iron-free Ybt to YbtA has not been demonstrated. The model is reproduced with modifications from Miller *et al* [31] with the permission of the Society for General Microbiology. (C) YbtA binding sites in the *irp2*, *ybtP*, *ybtA*, and *psn* promoter regions. Repeat sequences (RSs) are involved in YbtA binding. RS1, RS2, and RS3 as defined by

Animisov *et al* are denoted in red, uppercase text [57–58]. Extended RS (overlined uppercase text) are proposed based on comparison of RSs among the *ybt* promoters. Underlined text indicates −35 regions of the promoter regions. The lowercase blue text below the *psn* sequence shows the mutated sequence that lowered expression of a *psn::lacZ* reporter [53].