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Zinc transporters YbtX and ZnuABC are required for the virulence of *Yersinia pestis* in bubonic and pneumonic plague in mice

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Abstract

A number of bacterial pathogens require the ZnuABC Zinc (Zn^{2+}) transporter and/or a second Zn^{2+} transport system to overcome Zn^{2+} sequestration by mammalian hosts. Previously we have shown that in addition to ZnuABC, *Yersinia pestis* possesses a second Zn^{2+} transporter that involves components of the yersiniabactin (Ybt), siderophore-dependent iron transport system. Synthesis of the Ybt siderophore and YbtX, a member of the major facilitator superfamily, are both critical components of the second Zn^{2+} transport system.

Here we demonstrate that a *ybtX znu* double mutant is essentially avirulent in mouse models of bubonic and pneumonic plague while a *ybtX* mutant retains high virulence in both plague models. While sequestration of host Zn is a key nutritional immunity factor, excess Zn appears to have a significant antimicrobial role in controlling intracellular bacterial survival. Here, we demonstrate that ZntA, a Zn^{2+} exporter, plays a role in resistance to Zn toxicity *in vitro*, but that a *zntA zur* double mutant retains high virulence in both pneumonic and bubonic plague models and survival in macrophages. We also confirm that Ybt does not directly bind Zn^{2+} *in vitro* under the conditions tested. However, we detect a significant increase in Zn^{2+}-binding ability of filtered supernatants from a Ybt^{+} strain compared to those from a strain unable to produce the siderophore, supporting our previously published data that Ybt biosynthetic genes are involved in the production of a secreted Zn-binding molecule (zincophore). Our data suggest that Ybt or a modified Ybt participate in or promote Zn-binding activity in culture supernatants and is involved in Zn acquisition in *Y. pestis.*

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Introduction

Zinc (Zn) is an essential transition metal for archaeal, bacterial, and eukaryotic organisms where it serves both catalytic and structural functions.[1-4] In recent years, the definition of metal nutritional immunity has been expanded to include zinc ions, Zn^{2+}, (and manganese [Mn^{2+}] as well as other biometals and organic compounds) as mammalian hosts attempt to restrict the availability of Zn^{2+} to invading pathogens. Serum Zn levels are chelated by a variety of proteins and this metal is further sequestered, both locally and systemically upon infection.[5-16]

Consequently, like iron (Fe), bacterial high affinity Zn^{2+} uptake systems are important for the virulence of a number of bacterial pathogens.[17-40] However, only a relatively small number of different uptake mechanisms have been identified in these pathogens. The ZnuABC family of ABC transporters is the most common with a second periplasmic Zn^{2+}-binding protein, ZinT, working through the Znu system in some bacteria in conjunction with ZnuA, the primary periplasmic Zn^{2+}-binding protein of the ZnuABC system.[2, 41-43] Although described as a low-affinity Zn^{2+} transporter, ZupT, a ZIP family proton motive force-dependent transporter, functions at micromolar Zn^{2+} levels *in vitro*, and plays a role in the virulence of *Escherichia coli* UPEC and *Salmonella*.[21, 28, 38, 44] Two additional inner membrane (IM) transporters (ZevAb and ZurAM) have been identified in *Haemophilus influenzae* and *Listeria monocytogenes*, respectively.[22, 37]

Recently, we demonstrated that HMWP2 (encoded by *irp2*), a nonribosomal peptide synthetase required for synthesis of the yersiniabactin (Ybt) siderophore, and the putative IM protein YbtX are both involved in Zn^{2+} uptake in *Yersinia pestis,* the causative agent of bubonic, septicemic, and pneumonic plague.[45] *In vitro,* single *irp2::kan* and *Δirp2* mutants (KIM6-2046.1 and KIM6-2046.3, respectively) have no growth defect in the Chelex-100 treated defined medium PMH2 (cPMH2; residual Fe and Zn^{2+} concentrations of ~0.3 and ~0.5 μM, respectively) while a single *ΔznuBC* mutant (KIM6-2077+) has a significant growth defect in this medium. The *irp2::kan ΔznuBC* double mutant (KIM6-2077.7) is unable to grow in cPMH2 unless supplemented with Zn^{2+} at 2.5 μM. Intriguingly, YbtX, a member of the Major Facilitator Superfamily (MFS), but not Psn, TonB, or YbtPQ (required for Fe^{3+} uptake *via* Ybt) is required for Ybt-dependent acquisition of Zn^{2+}. Finally, single *irp2::kan* and *ΔznuBC* mutants retain high virulence in a mouse model of septicemic plague while the *irp2::kan ΔznuBC* double mutant shows a > 4.3×10^5-fold virulence loss in this model.[45]

While secreted Zn^{2+}-chelating compounds (zincophores) have been proposed as a Zn^{2+}-acquisition mechanism for pathogens,[8] examples of Zn^{2+}-binding by secreted compounds and subsequent delivery to the bacterial cell for nutritional use are limited. The 33-kDa secreted Pra1 protein of the fungal pathogen *Candida albicans* binds Zn^{2+} and a *pra1Δ* mutant has reduced hyphal growth on endothelial cells that is alleviated by Zn^{2+} supplementation.[46] The plant pathogen *Pseudomonas putida* produces a small siderophore, pyridine-2,6-bis(thiocarboxylic acid) (PDTC) that binds Fe^{3+} and Zn^{2+} and delivers these cations to the bacterial cell.[47, 48] *Streptomyces coelicolor* produces a siderophore-like

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molecule, coelibactin, which has been proposed, but not proven, as a zincophore.[49, 50] In addition, the *Pseudomonas aeruginosa* siderophores pyochelin and pyoverdin bind a variety of cations, including Zn^{2+}, but nutritional delivery to the bacterial cell has not been tested. Instead, it has been proposed that this metal chelation may promote resistance to metal toxicity.[51-53] Finally, *Pseudomonas* spp and *Ralstonia solanacearum* produce micacocidin, a compound with a chemical structure similar to Ybt, which has been crystallized with Zn^{2+}, Mn^{2+}, and Fe^{3+}.[54, 55]

Here we show the importance of ZnuABC and YbtX in Zn^{2+} acquisition in mouse models of bubonic and pneumonic plague and investigate the mechanism of Ybt-dependent Zn^{2+} uptake in *Y. pestis.* Finally, while Zn toxicity plays a role in controlling intracellular bacterial survival, we show that a Δ*zntA zur::kan* mutant retains high virulence in mouse models of bubonic and pneumonic plague as well as survival in macrophages. In contrast, ZntA plays an important role in resistance to Zn toxicity *in vitro*.

Experimental

Bacterial strains, plasmids, primers and growth conditions

The bacterial strains, plasmids and primers used in this study are listed in Table S1 in supplemental material. *E. coli* strains DH5α and DH5α (λ*pir*) were used in construction and maintenance of recombinant plasmids and were grown in Luria broth (LB) or on LB agar at 28-37°C. For *Y. pestis* KIM strains, a plus sign indicates an intact chromosomal 102-kb *pgm* locus. All other *Y. pestis* strains have a mutation within this locus or a deletion of the entire locus. Genes which encode for the synthesis and transport of Ybt as well as transcriptional regulation of *ybt* genes are encoded within the *pgm* locus. *Y. pestis* avirulent strains lacking the pCD1 virulence plasmid were used for construction of mutants and for *in vitro* studies. The *Y. pestis* strains were grown in autoclaved Heart Infusion Broth (HIB), Brain Heart Infusion (BHI) or on Tryptose Blood Agar Base (TBA) (Difco) at 26-33°C. For some experiments, HIB that was sterilized by passage through 0.22 μm filters (Millipore) (HIB-FS) was used. Where necessary, ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), streptomycin (Sm), or tetracycline (Tc) were routinely used at final concentrations of 100, 30 or 15, 50, 50, and 12.5 μg ml^{-1}, respectively, unless noted otherwise.

For metal-deficient growth studies glassware was cleaned with a chromic-sulfuric acid solution to remove contaminating metals as previously described.[56] Metal-deficient, chemically defined media PMH or PMH2 (cPMH or cPMH2, respectively) were prepared as described previously using Chelex-100.[56-58] *Y. pestis* strains were inoculated from -80°C glycerol stocks onto TBA supplemented with 0.2% galactose, Congo red (TBA-CR) and $ZnSO_4$ or $ZnCl_2$ at final concentrations of 100 μg ml^{-1} and 10 μM, respectively and grown at 32-33°C for 2 days. Cells which bind CR have retained the *pgm* locus. CR positive cells (red colonies) were grown on TBA slants at 32-33°C overnight and then inoculated to an OD_{620} of ~0.1 in cPMH2 with $ZnCl_2$ and $FeCl_3$ added to final concentrations of 0.6 μM and 1 μM where indicated, respectively. Growth of the cultures was monitored by determining the OD_{620} with a Genesys5 spectrophotometer (Spectronic Instruments, Inc.).

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For Zn toxicity assays, *Y. pestis* cultures grown in HIB-FS medium were back diluted to an OD_{620} of ~0.025 in HIB-FS containing a range of $ZnSO_4$ concentrations from 0 to 800 μM. Cultures were grown overnight and the final OD_{620} was measured. In all assays, bacteria were grown at 37°C with shaking.

Mutant strain construction

Suicide plasmids pSucZnu3.5, pKNGΔznuA, and pZur4 were used to introduce mutations *ΔznuBC*, *ΔznuA*, and *zur::kan* respectively, into various Y. pestis KIM strains by allelic exchange and confirmed by PCR as described previously.[45, 59] TBA medium supplemented with 5% sucrose (TBAS) was used to excise integrated suicide vectors expressing SacB. Plasmid isolation and sensitivity to the appropriate antibiotic was used to confirm loss of the suicide vectors. All bacterial strains, plasmids and primers used in this study are listed in Table S1 in supplemental material.

To replace *zntA* with a *cam* casette, Y0410red-1 and Y0410red-2 primers were used to prepare a PCR product from pKD3 which was transformed into KIM6(pWL204)+; the *ΔzntA::cam2196* mutation was verified with primers Y0410-pBAD-F and Cm-2 (Table S1). The *cam* casette was removed using pSkippy[60] and confirmed by demonstrating that the resulting strain was now Cm sensitive. Growth on TBAS plates was used to cure strains of pWL204 and pSkippy and their loss was confirmed by plasmid analysis and restoration of antibiotic sensitivities. Using suicide vector pZur4, zur^+ was inactivated in the *ΔzntA* mutant (KIM6-2196.1+). The presence of the *zur::kan2078* mutation in the resulting *ΔzntA zur::kan* strain, designated KIM6-2196.4+, was verified by PCR using primers zur3.3 and zur5.3 as described previously.[59]

The gene *y3657* was replaced with a *kan* cassette from pKD4 in KIM6-2077+ (*ΔznuBC*). The PCR product was prepared using Y3657red-F and Y3657Rred-R, transformed into KIM6-2077(pWL204)+ and Km^r colonies were streaked onto TBAS plates to eliminate pWL204. The *Δy3657::kan2203* mutation was verified by PCR using primers KM-1 and Y3657 del. One confirmed mutant was named KIM6-2202.1+ (*Δy3657::kan ΔznuBC*) and used in subsequent experiments.

Repair of the *ybtX* mutation

An ~ 2.64 kb fragment from the *ybtX* region of *Y. pestis* KIM10+ genomic DNA was amplified with Phusion HF polymerase (New England Biolabs) using primers YbtX-comp-*Hin*dIII and YbtX-comp-Rev. After purification and digestion with *Hin*dIII, the PCR product was cloned into the *Hin*dIII and *Sma*I sites of pWSK29 to generate pWSK-YbtX-comp. After the presence of the correct insert was confirmed by sequencing (ACGT) using primers pPQX-vector-2100 and pPQX-vector-1300, the plasmid was digested with *Apa*I and *Bam*HI and a 2.67 fragment containing $ybtX^+$ was ligated into the *Apa*I and *Bam*HI sites of pKNG101 generating pKNG-YbtX-comp. This suicide vector was electroporated into the *ΔybtX ΔznuA* mutant (KIM6-2197.2). Incubation on TBAS plates selected for a strain in which the in-frame *ΔybtX2067* mutation was replaced by $ybtX^+$ resulting in the $YbtX^+$ Znu^- strain KIM6-2197.4+ ($ybtX^{rp}$ *ΔznuA*). The pWSK-YbtX-comp plasmid was also digested with XhoI and *Bam*HI and a 2.66-fragment ligated into *Sal*I and *Bam*HI sites of pSR47s

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generating pSR-YbtX-comp. In the BSL3 facility, this suicide plasmid was also used to restore *ybtX*$^{+}$ in KIM5-2197.2 (pCD1Ap), a *ΔybtX ΔznuA* mutant carrying the virulence plasmid that encodes a type three secretion system, generating KIM5-2197.4(pCD1Ap)+ (*ybtX*^{tp} *ΔznuA*). The restoration of *ybtX*$^{+}$ in both strains was confirmed with primers P27 and P33.

Cloning of *ybtX* expressed from the *znuA* promoter

Primers ybtXcompl_R6K_R-SpeI and ZnuAprom-ybtX-BamHI (which includes 63 bp of the *znuA* promoter region and 23 bp containing a putative Shine-Dalgarno sequence upstream of the predicted translational start of *ybtX* ORF) were used to generate an ~ 1.6-kb fragment that contains the coding sequence for *ybtX* cloned downstream of the *znuA* promoter using Phusion HF polymerase (New England Biolabs) and the pYbtX plasmid [45] as a template. After purification, the PCR product was digested with *Spe*I and *Bam*HI and ligated into the *Xba*I and *Bam*HI sites of pACYC184 to generate pYbtX-ZP. The presence of the correct fragment was confirmed by sequencing (ACGT Inc.) and the plasmid was electroporated into KIM6-2097.1 (*Δirp2 ΔznuA*).

Western Blot Analysis

Cells were grown at 37°C under metal-deficient conditions for two transfers, pelleted at 6000 × g for 10 minutes, resuspended in SDS-sample buffer at a final OD_{620} of 10.0 and disrupted by vortexing with Zirconia beads. A 20 μl sample was loaded onto a 12.5% SDS gel and the proteins were electrophoresed at 80 V at room temperature. After electrophoresis, the gel was incubated in a carbonate transfer buffer (10 mM $NaHCO_3$, 3mM Na_2CO_3, pH 9.9, 20% methanol) for 10 minutes on ice and the proteins transferred to a PVDF membrane at 100 mA in an ice bath overnight.[61] A 1:1000 dilution of antiserum to YbtX was used for detection. Rabbit antiserum against YbtX was made to a peptide (YIRLHSARELMYSAID) in the C-terminus of the protein (Open Biosystems, Inc.).

Growth complementation with culture supernatants

Feeding assays with culture supernatants were performed as previously described.[45] Briefly, for supernatant preparation, cells were grown under metal-deficient conditions for two transfers (~6-8 generations), the cells pelleted and supernatants filter sterilized (0.22 μm Millipore). The *Y. pestis* recipient strain *irp2::kan Δpsn ΔznuBC* (KIM6-2077.18) was grown at 37°C in cPMH2 supplemented to 0.6 μM $ZnCl_2$ and 1 μM $FeCl_3$ for ~3-4 generations before diluting to an OD_{620} of ~0.1 in the same medium containing 50% (v/v) culture supernatants from test strains. Bacteria were grown overnight and the final OD_{620}s were measured.

Apo-Ybt purification

Apo-Ybt was isolated and purified in a multi-step procedure slightly modified from previous methods.[62, 63] No iron was added to the spent medium before extraction. Eight liters of spent medium were extracted three times in 500 ml aliquots with 200 ml portions of ethyl acetate. The organic layers were combined and the solvent was removed by rotary evaporation at

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40°C in the dark. The material was then dissolved in ~ 5 ml of absolute ethanol and then further diluted with 50 ml of 18 MΩ water for the next purification step.

The crude Ybt extract was dissolved in a 10:1 water-ethanol solution, loaded onto a 2.5 g pre-packed C_{18} reverse phase cartridge, and purified on a 4.3 g C_{18} reverse phase column with water and acetonitrile as the mobile phase using a Combiflash R_f. The separation was achieved by using the following gradient: 10% acetonitrile for 3 min followed by a linear gradient from 10% to 70% acetonitrile over 25 min (R_t for Yel ≈9 min and R_t for Ybt ≈14 min for the Combiflash purification). Fractions were collected at 1 min intervals. During HPLC/MS analysis, two apo-Ybt peaks with retention times of 7.8 and 8.3 min were observed. In addition, two peaks with retentions times of 5.6 and 5.9 min with the same *m/z* ratio of 297.07 were also observed; these truncated versions of Ybt are tentatively designated yersinol (Yel) and will be described in a separate study. Fractions containing apo-Ybt were combined and concentrated in the dark *via* rotary evaporation at 40°C. The purification protocol was repeated to afford apo-Ybt with excellent purity.

The identity and purity of compounds in each stage of purification were confirmed *via* mass spectrometry using an Agilent 6224 TOF LC/MS equipped with the Agilent 1260 HPLC system and MassHunter software. The instrument is equipped with an Agilent Extend C_{18} column (1.8 μm, 2.1 × 50 mm) with mobile phase consisting of mass spectrometry grade water (with 0.1% formic acid and 0.1% methanol) and acetonitrile (with 0.1% formic acid) and operated in positive ion mode (3500 V Vcap, 750 V OctRF Vpp, 65 V skimmer, 135 V fragmenter, 40 psi Nebulizer gas, 12 L min^{-1} drying gas, and 325°C gas temperature). Samples were eluted with a linear gradient of 5 to 100% acetonitrile at 0.3 mL min^{-1} over 15 min.

Apo-Ybt eluted as diastereometers (1.0:3.5) at approximately 7.8 min (minor) and 8.3 min (major). HRMS (ESI+): exact mass calculated for $C_{21}H_{28}N_3O_4S_3$ $[M+H]^+$, 482.1236 and for $C_{21}H_{29}N_3O_4S_3$ [M+2H]2+, 241.5655; found 482.1246 and 241.5664 for the minor isomer and 482.1239 and 241.5655 for the major isomer. In the original isolation and structural analysis of Ybt, Dreschel *et al* noted two apo-Ybt peaks which they characterized as epimers (a type of diastereomer) which are readily interconverted.[64] Our finding of two apo-Ybt peaks that coalesce into one Fe-Ybt complex[63] support the conclusions of Dreschel *et al.* that these peaks represent apo-Ybt epimers that are converted to one form by binding Fe^{3+}.[64] Thus, we have combined these two peaks for our analyses of apo-Ybt. The isolated apo-Ybt was further analyzed by comparing the LC/MS data of the isolated material to authenticated apo-Ybt and was found to be identical (data not shown).

PAR assay

Zn^{2+} binding assays were performed using the metallochromic indicator dye 4-(2-pyridylazo)-resorcinol (PAR) (Sigma) and the methodology of Hunt et al. [65] PAR has a low absorbance at 490-500 nm in the absence of Zn^{2+}; upon complexing with Zn^{2+} the absorbance at this wavelength increases dramatically. To determine Zn^{2+} chelating activity in *Y. pestis* supernatants, KIM6+ (Ybt^+) and KIM6-2046.1 (Ybt^-; *irp2::kan* mutant) were grown at 37°C in cPMH medium[58] supplemented with 40 mM MOPS pH7.5 for 30 h and supernatants harvested by pelleting cells. Cell densities of the two strains tested were

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equivalent. Supernatant aliquots of 0.2 ml were filtered and used in the PAR assay (total volume of 0.5 ml). Reactions containing 40 mM HEPES-KOH, pH 7.4 and 3 (culture supernatants alone) or 5 μM $ZnCl_2$ (*irp2::kan* mutant culture supernatants with or without 20 μM apo-Ybt) were incubated for 15 min at room temperature before a PAR solution at pH 7.0 was added to a final concentration of 50 μM. Residual Zn in cPMH is not sufficient to yield measurable chelation by PAR; consequently, any differences in Zn uptake between the two strains did not affect the assays, in which 3 uM $ZnCl_2$ was added prior to incubation with PAR. After five min of incubation, the absorbance at 497 nm was measured with a Genesys5 spectrophotomer (Spectronic Instruments, Inc).

Zn^{2+}-binding studies of Ybt by ^{1}H NMR spectroscopy

Briefly, a 6.6 mM solution of Ybt was first prepared by dissolving 1.9 mg of apo-Ybt in 600 μl of acetonitrile-d_3 (CD_3CN) and analyzed by ^{1}H NMR spectroscopy. It was then treated with 3 equivalents of a solution of $ZnCl_2$ in CD_3CN and allowed to stand for 3 min. The resulting mixture was also analyzed by ^{1}H NMR spectroscopy as previously described.[66]

Cu^{2+}, Fe^{3+} and Zn^{2+}-binding studies with Ybt

A 100 mM master stock solution of $CuCl_2$ was prepared by dissolving 0.60 g copper (II) chloride dihydrate powder (Sigma Aldrich) in 35.2 ml of ddH_2O. A 2-fold dilution of this master stock solution yielded a 50 mM $CuCl_2$ stock solution, C_1. A 100 mM master stock solution of $FeCl_3$ was prepared by dissolving 0.73 g iron-(III) hexahydrate in 27.0 ml mQH_2O. A 2-fold dilution of this master stock solution yielded a 50 mM $FeCl_3$ stock solution, C_2. A 1.00 M master stock solution of $ZnCl_2$ was prepared by dissolving 0.683 g $ZnCl_2$ in 5 ml of ddH_2O. A 2-fold dilution of this master stock solution yielded a 500 mM $ZnCl_2$ stock solution, which was further subjected to a 10-fold dilution to yield a 50 mM $ZnCl_2$ stock solution, C_3. To a cuvette containing 950 μl of a 0.526 mM solution of Ybt in EtOH was added 50 μl of H_2O, C_1, C_2 or C_3 corresponding to treatment with 0 or 5 equivalents of $CuCl_2$, $FeCl_3$, or $ZnCl_2$, respectively. The resulting solution was gently mixed and allowed to stand for 2 min at room temperature before its absorbance was measured by UV-Vis spectroscopy from 200 to 900 nm in 10 nm increments as previously described.[67]

Virulence testing

Construction and testing of potentially virulent strains was performed in a CDC-approved, BSL3 laboratory following Select Agent regulations using procedures approved by the University of Kentucky Institutional Biosafety Committee. All animal care and experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

For Y. pestis KIM6-2067(*ΔybtX*), KIM6-2197.2 (*ΔybtX ΔznuA*), KIM6-2070.3 (*ybtS::kan ΔznuBC*), and KIM6-2196.4+ (*ΔzntA zur::kan*) mutants and KIM6-2197.4+ ($YbtX^+$ Znu^-; $ybtX^{rp}$ [repaired *ΔybtX*] *ΔznuA*), pCD1Ap was electroporated into each strain resulting in strains KIM5-2067(pCD1Ap) (*ΔybtX*), KIM5-2197.2(pCD1Ap) (*ΔybtX ΔznuA*), KIM5-2070.3(pCD1Ap) (*ybtS::kan ΔznuBC*), KIM5-2196.4(pCD1Ap)+ (*ΔzntA zur::kan*) and KIM5-2197.4(pCD1Ap)+ ($ybtX^{rp}ΔznuA$). Plasmid profiles, type III secretion phenotype and expression levels, and the CR phenotype were assessed as described previously.[45, 57] For

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LD_{50} studies, CR positive colonies were grown on a TBA slant overnight at 26°C, inoculated to an OD_{620} of ~0.1 in HIB or HIB-FS supplemented with 50 μg Ap ml^{-1}, 0.2% xylose, 2.5 mM $CaCl_2$ and 10 μM $ZnCl_2$ (except *ybtS::kan* and *ΔzntA zur::kan* mutants which were grown without Zn^{2+} supplementation) and grown at 26°C (for subcutaneous infections) or 37°C (for retro-orbital or intranasal infections) overnight. These cultures were diluted to an OD_{620} of ~ 0.1 into the same medium, but without zinc supplementation, and grown at the same temperature for approximately two generations. 10-fold serially diluted bacterial suspensions[45, 57] of *ΔybtX*, *ΔybtX znuA, ybtX*^{rp}*ΔznuA,* and *ΔzntA zur::kan* mutants were used for subcutaneous injections or intranasal infections of 6- to 8-week-old female Swiss Webster mice (Hsd::ND4).10-fold serial dilutions of *ybtS::kan* and *ybtS::kan ΔznuBC* mutants were injected into the retro-orbital plexus of Swiss Webster mice. For intranasal and retro-orbital infections, mice were sedated by intraperitoneal injection of a mixture of 100 μg of ketamine and 10 μg of xylazine per kg of body weight. Appropriate serial dilutions of suspensions used for injections were inoculated onto TBA plates containing Ap (50 μg ml^{-1}) and 10μM $ZnCl_2$ (Zn supplementation was omitted for *ybtS::kan* and *ΔzntA zur::kan* mutants) and colonies were counted after 2–3 days of incubation at 26°C. Mice were observed daily for 2 weeks and LD_{50} values were calculated according to the method of Reed and Muench.[68]

Macrophage infection and survival

Peritoneal macrophages were isolated from C57/Bl6 mice injected with 3 ml of thioglycolate medium as previously described.[69] Peritoneal and RAW264.7 macrophages were maintained in DMEM + 10% FBS. For infections, *Y. pestis* was grown overnight at 26°C in BHI, diluted 1:25 in fresh BHI, and grown at 26°C to an OD_{600} ~1.0. Bacteria were diluted to desired concentration in 37°C DMEM+10%FBS and added to macrophages at a MOI=10. The infection was synchronized by centrifugation and extracellular bacteria were killed with 8 μg/ml gentamicin 20 min post-infection. One hour after gentamicin treatment, the medium was replaced with DMEM + 10% FBS containing gentamicin at a concentration of 2μg/ml (RAW264.7) or 1μg/ml (peritoneal). Intracellular bacterial numbers were determined by conventional CFU enumeration in triplicate as previously described.[70] Three independent trials with RAW274.6 cells were performed; one representative trial is shown. Patterns for RAW274.6 cells (growth) and peritoneal macrophages (survival but not growth) were typical for *Y. pestis*. The one trial with peritoneal macrophages confirmed the lack of a mutant phenotype similar to the RAW274.6 trials.

Results

The role of zinc homeostasis in the lethal progression of bubonic and pneumonic plague in mice

Previously, we demonstrated the critical role of *Y. pestis* Zn^{2+} uptake via a HMWP2 product and the ZnuABC transporter for the lethal progression of septicemic plague in mice using an *irp2::kan ΔznuBC* mutant (KIM5-2077.7(pCD1Ap). Ybt biosynthetic mutants cannot be used in bubonic and pneumonic plague models since this mutation alone causes dramatic virulence losses (*e.g.*, *irp2* mutations show >5 × 10^5-fold and 790-fold virulence losses, respectively).[57]

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However, previous studies showed that YbtX is not required for Fe^{3+} uptake but rather for Ybt-dependent Zn^{2+} uptake;[45, 71] thus we used single *ΔybtX* or *ΔznuA* mutants, a double *ΔybtX ΔznuA* mutant and a *ybtX*^{rp} (repaired *ΔybtX*) *ΔznuA* mutant carrying the pCD1Ap virulence plasmid that encodes a type three secretion system [KIM5-2067(pCD1Ap) (ΔybtX) or KIM5-2197(pCD1Ap)+ (*ΔznuA*), KIM5-2197.2(pCD1Ap) (*ΔybtX* ΔznuA) and KIM5-2197.4(pCD1Ap)+ (*ybtX*^{rp}*ΔznuA*), respectively] to assess the role of these systems in Zn^{2+} acquisition in mouse models of bubonic and pneumonic plague. Outbred Swiss-Webster mice were infected subcutaneously or intranasally to mimic bubonic or pneumonic plague, respectively, and observed for two weeks. As expected, the *ΔybtX* mutant retained high lethality similar to the parent strain and the *ΔznuB* mutant (Table 1and Ref [59, 72]). However, the double *ΔybtX ΔznuA* mutant was essentially avirulent in mouse models of bubonic and pneumonic plague (Table 1). The *ΔybtX ΔznuA* mutant showed an ~10^6-fold loss of virulence compared to the *ΔybtX* strain in the mouse model of bubonic plague. Strikingly, intranasal instillation with 1.5×10^6 cells (highest infectious dose used) of the double mutant did not cause any disease symptoms, suggesting complete attenuation. The virulence of the *ΔybtX ΔznuA* mutant was restored in both models when the chromosomal *ΔybtX* mutation was repaired (*ybtX*^{rp}*;* Table 1). These data show that Zn^{2+} acquisition is essential for the progression of both bubonic and pneumonic plague. While individually the *Y. pestis* YbtX and ZnuABC transporters are dispensable, mutation of both systems causes essentially complete attenuation in both bubonic and pneumonic plague mouse models .

While bacteria require Zn for growth, elevated levels of Zn can also be toxic to bacteria and phagocytes have evolved to use Zn as part of their antimicrobial arsenal.[73-77] Therefore, the ability to avoid Zn toxicity via Zn^{2+} efflux has also been shown as an important virulence factor in several bacteria.[76, 78-80] *Y. pestis* is a facultative intracellular pathogen and has been shown to survive in macrophages, and to a lesser extent in neutrophils.[81-85] While bioinformatics identified several systems predicted to be involved in Zn^{2+} efflux in the *Y. pestis* KIM10+ genome,[59] their role in virulence and in preventing Zn toxicity has not been determined. Y410 encodes a protein with a high degree of similarity (65% identity over 709 amino acid residues) to ZntA, a P-type ATPase that is a primary Zn^{2+} exporter in *E. coli*.[86] Transcription of *Y. pestis zntA* was detected in the lymph nodes of plague-infected rats but not during colonization of fleas,[87, 88] suggesting that zinc efflux by ZntA may be important during bubonic plague. Deletion of *zntA* (KIM6-2196.1+) led to drastically increased Zn sensitivity *in vitro* compared to the parent strain (Fig. 1) suggesting that ZntA plays an important role in preventing Zn toxicity in *Y. pestis*. Another mechanism to prevent Zn toxicity in bacteria occurs via transcriptional repression of genes encoding Zn^{2+} importers by Zur.[2, 89] In *Y. pestis*, inactivation of Zur leads to increased expression of *znuABC* genes[59, 90] which causes unregulated Zn^{2+} uptake and could result in increased susceptibility to toxic Zn levels. However a *zur::kan* mutation (strain KIM6-2078) did not affect sensitivity of *Y. pestis* to high levels of Zn^{2+} (Fig. 1). The sensitivity of a double *ΔzntA zur::kan* mutant (KIM6-2196.4+) to Zn was similar to that of the *ΔzntA* mutant (Fig. 1). These results suggest that ZntA is important in preventing Zn toxicity while Zur is not – at least *in vitro.*

To assess the *in vivo* role of ZntA and Zur, we preformed subcutaneous (bubonic plague) and intranasal (pneumonic plague) infections using the double *ΔzntA zur::kan* mutant transformed with pCD1Ap to restore potential virulence [KIM5-2196.45(pCD1Ap)]. The

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double mutant had an LD_{50} similar to that for the parent Zur^+ $ZntA^+$ strain [KIM5(pCD1Ap)+] in the bubonic plague model (Table 1 and Ref [57]). In the mouse model of pneumonic plague, the double *ΔzntA zur::kan* mutant showed only a slight (~4-fold) increase in the LD_{50} compared to that of the parent strain (Table 1 and Ref [57]).

Since the Zn toxicity host defense occurs in phagocytic cells,[73-77] we tested the survival of the *ΔzntA zur::kan* mutant in RAW274.6 and peritoneal macrophages. Over a 24-h period post-infection, the parent ($ZntA^+$ Zur^+ KIM6+) strain and the *ΔzntA zur::kan* mutant (KIM6-2196.4) had nearly identical invasion, survival and growth characteristics in both RAW274.6 and peritoneal macrophages (Fig. 2). Together, these results suggest that *Y. pestis* either does not face toxic Zn levels during mammalian infection or that other Zn^{2+} exporter(s) or Zn^{2+} homeostasis mechanisms compensate for the loss of ZntA and Zur in *Y. pestis*.

The salicylate synthase YbtS is irrelevant for Zn-dependent growth *in vitro* but critical for virulence of a *znu* mutant during septicemic plague

Previously, we showed that HMWP2 and YbtU (a reductase) are both required for Ybt biosynthesis and for growth under low Zn^{2+} conditions in a *znu* background.[45] Here we examine the effect of three other mutations that prevent Ybt biosynthesis, Δ*ybtE*, *irp2*Δ*S52* and *ybtS::kan,* on the growth, under low Zn^{2+} conditions, of a *ΔznuBC Y. pestis* strain. YbtE adenylates salicylate for loading onto residue S52 of HMWP2 and both the *ΔybtE ΔznuA* and the *irp2*Δ*S52 ΔznuA* double mutants (KIM6-2056.2 and KIM6-2046.9, respectively) were unable to grow in cPMH2 supplemented with 0.6 μM $ZnCl_2$ and 1 μM $FeCl_3$ (Fig. S1). In contrast, the *ybtS::kan* mutation in KIM6-2070.1 that inactivates the salicylate synthase YbtS did not affect growth in the *ΔznuBC* mutant background (KIM6-2070.3) under similar conditions (Fig. 3A).

Most mutations that abrogate Ybt biosynthesis cause reduced transcription of the *ybt* operons and thus lower expression of Ybt biosynthetic and transport enzymes. However in the *ybtS::kan* mutant, the expression level of the *ybt* genes is normal.[63, 91, 92] Previously, we speculated that a Ybt-like molecule produced by the remaining Ybt biosynthetic module in a *ybtS::kan* mutant was responsible for transcriptional activation of *ybt* genes but was not able to stimulate growth under iron-deficient conditions.[63] It is possible that this Ybt-like molecule also binds Zn^{2+} and thus permits the growth of the *ybtS::kan ΔznuBC* mutant. However, supernatants from a *ybtS::kan* and *irp2::kan* (negative control) cultures failed to stimulate the growth of the irp2::kan Δpsn ΔznuBC mutant (KIM6-2077.18) in contrast to the growth stimulation observed in supernatant from the Ybt^+ parent KIM6+ strain (Fig. 3B)

We next tested the virulence of the *ybtS::kan ΔznuBC Y. pestis* mutant in a septicemic mouse model of plague. Since the *ybtS::kan ΔznuBC* mutant had a growth phenotype similar to the *ΔznuBC* mutant (KIM6-2077+) *in vitro,* we expected this double mutant to have the same fully virulent phenotype as the single *ΔznuBC* mutant [KIM5-2197(pCD1Ap)]. Surprisingly, the *ybtS::kan ΔznuBC* mutant transformed with pCD1Ap [KIM5-2070.3(pCD1Ap)] was essentially avirulent with an ~10^6-fold loss of virulence compared to the *ΔznuBC* mutant (Table 1) – a virulence defect similar to that of an *irp2::kan ΔznuBC* mutant [KIM5-2077.7(pCD1Ap)].[45]

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Investigation of the siderophore-dependent mechanisms of Zn^{2+} acquisition in *Y. pestis*

Our earlier study showed that loss of Ybt biosynthesis (*Δirp2* or *irp2::kan* mutations) in combination with a *ΔznuBC* mutation caused an *in vitro* growth defect under low-Zn^{2+} conditions and a significant reduction in virulence in a mouse septicemic plague model. Supplementation of a *Δirp2 Δpsn ΔznuBC* culture with apo-Ybt stimulated growth *in vitro*.[45] The Henderson research group had previously shown that Ybt binds Cu^{2+}.[93] In a more recent study, this same research group found that purified Ybt also binds Cr^{3+}, Co^{2+} and Ni^{2+} but not Zn^{2+} or Mn^{2+}.[94] However, it is possible that *Y. pestis* produces isomeric form of Ybt with different metal binding properties. Note that two isomers of the pyochelin siderophore are transported by different transport systems in *P. aeruginosa* and *P. fluorescens.* [95] Therefore, we have investigated whether Ybt purified from *Y. pestis* directly binds Zn^{2+} and other possible roles of Ybt biosynthesis in Zn^{2+} uptake by *Y. pestis*.

In *Y. pestis*, the Ybt siderophore has a dual function as a Fe^{3+} carrier and as a signaling molecule that activates transcription of *ybt* operons including *ybtPQXS*. To determine whether the role of the siderophore is simply to stimulate expression of YbtX, we constructed a *ybtX* clone whose expression is controlled by the *znuA* promoter and examined the low-Zn^{2+} growth response of a double *Δirp2 ΔznuA* mutant (KIM6-2197.1) carrying this clone. Although the level of YbtX expressed was similar to that of a Ybt-producing strain, the growth of the mutant strain was not restored under these conditions (Fig. 4). Thus Ybt biosynthesis does not enhance Zn^{2+} uptake simply by increased expression of YbtX.

However, this does not eliminate the possibility that YbtA-Ybt activates transcription of an unidentified transport component that acts in conjunction with YbtX. To address this, we used the IPTG-inducible YbtA expression vector, pQEYbtA which we have previously shown activates transcription from the *ybtPQXS* promoter 42-fold even in the absence of the Ybt siderophore, similar to other overexpressed AraC-type transcriptional regulators.[92] The double *Δirp2 ΔznuA* mutant failed to grow in cPMH2 supplemented with 1 μM $FeCl_3$, 0.6 μM $ZnCl_2$, and 1 mM IPTG, whether this mutant carried the pQE30 vector or pQEYbtA (Fig. S2). Thus our data support a model in which Ybt is <u>not</u> simply serving as a signaling molecule to stimulate expression of YbtX and/or any additional putative Zn^{2+} transport components.

To test the ability of culture supernatants from a Ybt-producing strain (KIM6+) and a Ybt biosynthetic (*irp2::kan*) mutant (KIM6-2046.1) to chelate Zn^{2+}, we used the PAR assay which has been widely used for the identification of Zn-binding activity by a number of proteins. PAR is calculated to form 1:1 and 2:1 complexes with Zn^{2+} with stepwise affinity constants of 4.0×10^6 and 5.5×10^5 M^{-1}, which corresponds to an overall conditional stability constant of 2×10^{12} M^{-1}.[65] Therefore, the PAR assay can be used for monitoring Zn^{2+} binding in the range of nM to pM. To measure compounds with high affinity for binding Zn^{2+}, a PAR/Zn ratio of >10 was used to ensure the presence of only PAR_2-Zn complexes in the assay. If Ybt binds Zn^{2+}, it will remove free Zn^{2+} from the assay and reduce Zn^{2+} binding by PAR as measured by a reduction in absorbance at 497 nm. Indeed the culture supernatant from the Ybt-producing strain had a significantly higher Zn^{2+}-chelating activity compared to the supernatant from the *irp2::kan* mutant that is unable to

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produce Ybt (1.6-fold), and a 2.9-fold difference from uninoculated cPMH (Fig. 5A). Thus our data demonstrate the presence of a ligand(s) with high affinity to Zn^{2+} in the culture supernatant from the Ybt producing strain of *Y. pestis.*

Although the higher Zn^{2+}-binding activity detected in the Ybt-producing supernatant by the PAR assay (Fig. 5A) suggests that Ybt binds Zn^{2+}, Koh *et al.* found no evidence for Zn^{2+} binding by *E. coli* apo-Ybt by MS/MS analysis.[94] We have purified apo-Ybt from *Y. pestis* to further analyze its potential for Zn^{2+} binding. Addition of apo-Ybt to the *irp2::kan* culture supernatant did not change its Zn^{2+}-binding activity as determined by the PAR assay (Fig. 5B), a puzzling result since supernatants from Ybt-producing cells showed increased Zn^{2+}-binding activity compared to culture supernatants from a strain unable to synthesize Ybt. Consequently, we used additional approaches to test for Zn^{2+}-binding activity by Ybt. Similar to the results of Koh *et al.*,[94] our MS analysis of HPLC-purified apo-Ybt from *Y. pestis* showed no evidence of Zn^{2+} binding (data not shown). Addition of Fe^{3+} or Cu^{2+} significantly altered the UV-visible spectra compared to apo-Ybt (Fig. 6). In contrast, the absorption spectra of apo-Ybt compared to apo-Ybt plus Zn^{2+} were nearly identical (Fig. 6). While some small Zn^{2+}-binding molecules display differences in spectra upon Zn^{2+} binding,[96] our experience with chalcones indicates that this does not always occur.[67] To further assess possible Zn^{2+}-binding by Ybt, ^{1}H NMR spectra were performed. Using this approach, we found no significant differences in the ^{1}H NMR spectra of apo-Ybt and apo-Ybt plus Zn (Fig. 7). While our data indicate that purified *Y. pestis* Ybt does not directly bind Zn^{2+} in biochemical assays, it also suggests that Ybt or a modified Ybt participates in or promotes Zn-binding activity in culture supernatants and is involved in Zn acquisition in *Y. pestis.*

Discussion

Mammals respond to bacterial infections by increasing Zn^{2+} sequestration in an attempt to withhold Zn^{2+} from the invading pathogen (nutritional immunity) and a number of bacterial pathogens lose virulence when Zn^{2+} transporters are mutated, especially ZnuABC.[13-16, 97] Previously we found that a *Y. pestis ΔznuBC* mutant retained high virulence in both bubonic and pneumonic models of plague, suggesting that other Zn^{2+} transporter(s) compensated for the loss of ZnuABC. Mutation of a number of other cation transporters failed to identify any as Zn^{2+} transporters.[59, 98, 99] However, we found that an *irp2* mutation (causing loss of Ybt synthesis) in a *Y. pestis ΔznuBC* background has an extreme *in vitro* growth defect under low-Zn^{2+} conditions and was highly attenuated (> 4×10^5-fold virulence loss) in a mouse model of septicemic plague. We further found that YbtX, a member of the Major Facilitator Superfamily, is involved in Zn^{2+} acquisition. These results led us to suggest that Ybt might serve as a zincophore as well as a siderophore.[45]

While mutations that affect either transport or synthesis of Ybt cause nearly complete loss of virulence or high attenuation in bubonic and pneumonic models of plague, a *ybtX* mutation does not affect Fe^{3+} uptake via the Ybt system.[57, 71] Consequently, here we used a *Y. pestis ΔybtX ΔznuA* mutant [KIM5-2197.2(pCD1Ap)] to demonstrate nearly complete virulence loss in mouse models of bubonic and pneumonic plague due to these two mutations. Like the single *ΔznuBC* [in KIM5-2077(pCD1Ap)+][59] and Δ*znuA* mutations [KIM5-2197(pCD1Ap)

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+] (Table 1), a single *ΔybtX* mutation [in KIM5-2197(pCD1Ap)] did not significantly affect virulence in either disease model (Table 1 and Ref [72]). For a number of pathogens, including *Y. pestis,* the importance of specific $Fe^{3+ \text{ and } 2+}$ and Mn^{2+} transporters in disease progression or intracellular vs extracellular residence varies by organ system.[57, 97, 100-102] In contrast, these two Zn^{2+} transporters in *Y. pestis* appear to serve essential but overlapping or redundant functions *in vivo* with loss of both systems required to significantly affect virulence (Table 1 and Ref [59, 72]).

Since *ybtX* is highly expressed in the lungs during pneumonic infection [103], Pechous *et al.*[72] recently tested a *Y. pestis ybtX* mutant in a mouse model of pneumonic plague. They found that this mutation does not affect lung colonization or bacterial dissemination but reduces inflammation in the lungs likely by reducing expression of proinflammatory cytokines IL-6 and IL-17 and chemokines CXCL1 and CCL2 which decrease neutrophil infiltration. They speculated that YbtX reduces Zn levels in the lungs causing increased inflammation, a property of Zn^{2+} deficiency.[6, 15, 72, 75] Thus YbtX appears to serve two *in vivo* roles – contributing to bacterial Zn^{2+} acquisition and to inflammation in the lung.

While sequestration of host Zn^{2+} is a key nutritional immunity factor, there is recent evidence for excess Zn^{2+} having a significant antimicrobial role in controlling bacterial infections. Mutation of various Zn^{2+} exporters in several bacteria decrease survival in phagocytic cells and sometimes also affects the development of disease in mice.[75] Here we demonstrated that mutation of *zntA,* encoding a major Zn^{2+} exporter in *E. coli,* but not in *zur,* encoding a Zn-responsive transcriptional regulator, increased the sensitivity of *Y. pestis* to excess Zn *in vitro* (Fig. 1). However, a *Y. pestis ΔzntA zur::kan* double mutant [KIM5-2196.4([CD1Ap)] retained high virulence in mouse models of bubonic and pneumonic plague (Table 1) and resistance to killing by macrophages (Fig. 2). Although this suggests that *Y. pestis* does not encounter toxic levels of Zn^{2+} during mammalian infection, it is also possible that other putative Zn^{2+} exporters (*e.g.*, ZitB, ZntB and/or FieF) function *in vivo* to prevent toxicity.

Shortly after we suggested that Ybt might be a zincophore, the Henderson research group used MS analysis to demonstrate that Ybt purified from *E. coli* binds Cu^{2+}, Co^{2+}, Cr^{2+}, Ga^{3+} and Ni^{2+} but not Mn^{2+} or Zn^{2+}.[94] Here we examined the ability of apo-Ybt purified from *Y. pestis* to bind Zn^{2+} using MS (data not shown), ^{1}H NMR (Fig. 7), UV-Vis spectra (Fig. 6), and a Zn^{2+}-binding PAR assay (Fig. 5B). Results from all of these methods supported the original conclusion of Koh *et al.*[94] that apo-Ybt does not directly bind Zn^{2+} with significant affinity. These results are puzzling in light of our findings that: (1) Ybt does not appear to simply act as a signal molecule to increase expression of YbtX or other putative components of the Ybt-dependent Zn^{2+} transport system (Fig. 4 and S2); (2) the addition of apo-Ybt to the *irp2::kan Δpsn Δznu* mutant (KIM6-2077.18) stimulates growth under low-Zn^{2+} conditions;[45] and (3) supernatant from a Ybt-producing strain had a higher Zn^{2+}-binding activity by the PAR assay than supernatant from a non-producing strain (Fig. 5A). Detection by PAR indicates this unidentified Zn^{2+}-binding compound has a higher affinity for Zn^{2+} than PAR (2×10^{12} M^{-1}).

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Based on all these results, we favor two alternative models for Zn^{2+} acquisition using components of the Ybt system. First, Ybt plus a second compound (for convenience, tentatively termed YbtZ) together might bind Zn^{2+} (Fig. 8). Since addition of apo-Ybt to the growth medium stimulates growth of the double Zn^{2+} transport mutant, we expected that addition of apo-Ybt to the Ybt-negative culture supernatant would allow this interaction and cause increased Zn^{2+}-binding activity by the PAR assay. However, addition of apo-Ybt to the *irp2::kan* (KIM6-2046.1) culture supernatant did not change its Zn^{2+}-binding activity (Fig. 5B). Nonetheless, this scenario is still possible. Lability of the putative YbtZ in the absence of interaction with Ybt would explain why addition of apo-Ybt to a Ybt-negative culture supernatant did not restore Zn^{2+} binding. In this model secreted Ybt complexes with secreted YbtZ to form a zincophore (tentatively termed Zbt) (Fig. 8). In the second model, Ybt is enzymatically modified into a form that binds Zn^{2+}. In this model, conversion of Ybt to a Zn^{2+}-binding molecule (also termed Zbt) occurs after secretion (Fig. 8) since a *irp2::kan Δpsn ΔznuBC* mutant showed growth stimulation by external apo-Ybt. The *psn* mutation would prevent uptake and intracellular conversion of Ybt.[45, 104] Previously, we reported that a *Δpsn ΔznuBC* mutant grows better than a *ΔznuBC* mutant under Zn^{2+}-deficient conditions suggesting that the lack of a Psn receptor allows all available Ybt to be used for Zn^{2+} uptake.[45] These results fit with both models. However, isolation and identification of the putative Zbt zincophore (either Ybt-YbtZ or a modified Ybt) is needed to support either of these two proposed models. Although we have found that the Ybt synthesis-dependent Zn-binding activity is extracted into ethyl acetate (data not shown), we have yet to isolate the putative zincophore.

The Ybt-like compound produced by the *ybtS::kan* mutant (KIM6-2070.1; Fig. 3A and Ref [63]) remains elusive. We have not detected this compound in culture supernatants of the *ybtS::kan* mutant (Fig. 3B) which could indicate the compound is cell associated similar to some other siderophores.[105-108] Bioinformatics revealed several *Y. pestis* genes that could be involved in biosynthesis of a membrane-bound siderophore. In *Y. pestis, y2236* encodes a putative fatty acid CoA ligase orthologous to *V. harveyi* AebG, which is required for the synthesis of membrane-bound amphi-enterobactin.[106] Micacocidin, produced by a *Pseudomonas* sp. and *Ralstonia solanacearum*, binds Zn^{2+} and Fe^{3+} and has a structure very similar to that of Ybt with an additional pentyl chain on the salicylate moiety which we speculate could lead to cell association. This biosynthetic pathway does not use salicylate as a precursor[54, 55, 109, 110] and *Y. pestis* has a truncated gene (*y3406*) that encodes some domains of the polyketide synthase MicC from *Ralstonia solanacearum,* which initiates biosynthesis of micacocidin. MicC uses acyl carrier protein-tethered hexanoic acid as a precursor resulting in production of a hydrophobic pentyl chain linked to the salicylate moiety.[109] We speculated that a AebG-like or a MicC-related enzyme may initiate biosynthesis of a hydrophobic siderophore using the remaining Ybt biosynthetic machinery. However, neither the *Δy4306 ybtS::kan Δznu* (KIM6-2070.4) nor the *Δy2236 ybtS::kan ΔznuBC* (KIM6-2070.5) triple *Y. pestis* mutants had a growth defect similar to that of an *irp::kan ΔznuBC* double mutant (KIM6-2077.7) under low-Zn growth conditions (Fig. S3). While the pathway (and enzymes) for synthesis of the proposed Ybt-like molecule mutant remains to be identified, this compound seems unlikely to have a significant biological role

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during animal infections. The *ybtS::kan ΔznuBC* double mutant [KIM5-2070.3(pCD1Ap) was essentially avirulent in the mouse model of septicemic plague (Table 1).

The PAR assay detected a residual Zn^{2+}-binding activity in the Ybt-negative culture supernatant - a 1.8-fold difference compared to uninoculated cPMH (Fig. 5A). Athough this suggests that the *irp2::kan* mutant produces a secreted, high-affinity Zn^{2+}-binding compound, we have no evidence that this ligand plays a role in Zn^{2+} acquisition. Recently, two different extracellular Zn^{2+}-binding compounds have been characterized. Wang *et al.*[111] demonstrated that a 117-residue protein (YPK_3549) of *Yersinia pseudotuberculosis* binds Zn^{2+}. This protein, which was designated YezP (*Yersinia* extracellular Zn^{2+}-binding protein) is secreted by a type VI secretion system. Mutation of the secretion system reduced intracellular Zn levels *in vitro* and a *yezP znu Y. pseudotuberculosis* double mutant showed a significant loss of virulence via orogastric infection of mice.[111] *Y. pestis* encodes homologues of the type VI secretion system and *yezP* (*y3657* in *Y. pestis*). However, Y3657 is unlikely to be responsible for the residual Zn^{2+}-binding activity in the *znu irp2* mutant since our preliminary isolation studies of the unidentified Zn^{2+}-binding ligand indicates it is <3 kDa, resistant to boiling and proteinase K treatments (data not shown). In addition, we have no evidence that *y3657* plays a Zn^{2+} acquisition role in *Y. pestis.* The *irp2::kan Δznu* and *ΔybtX ΔznuA Y. pestis* double mutants are growth defective with 0.6 μM Zn supplementation (Fig. S3 and Ref[45]) suggesting no additional high affinity Zn^{2+} uptake system is functional *in vitro.* Also, a *Δy3657::kan ΔznuBC* double mutant (KIM6-2202.1+) had an *in vitro* growth phenotype similar to the *znu* mutant (data not shown). Thus, in *Y. pestis,* at least *in vitro,* Y3657 does not seem to be an independent Zn^{2+} importer or to work with the Ybt system for Zn^{2+} uptake.

The second system is a metallophore (staphylophine) and ABC transporter in *Staphylococcus aureus* that is involved in Co^{3+}, Cu^{2+}, Fe^{3+}, Ni^{2+} and Zn^{2+} acquisition. CntKLM enzymes synthesize the small metallophore while CntE exports it and CntA-F import the metal complex.[112, 113] The *Y. pestis* KIM genome has genes with significant similarities for the biosynthesis of the metallophore.[112] Consequently, the Ybt-independent Zn^{2+}-binding compound could correspond to staphylophine. However, our current evidence does not support an important *in vitro* or *in vivo* Zn^{2+} acquisition role for the staphylophine system in *Y. pestis.* Our *irp2::kan ΔznuBC* and *ΔybtX ΔznuA* double mutants are growth defective under low Zn^{2+} growth conditions *in vitro* and our *ΔybtX ΔznuA* double mutant is essentially avirulent in bubonic and pneumonic plague mouse models (Table 1 and Ref[45]). We have also examined the *Y. pestis* ABC transporter (*y2842-y2837*) encoded upstream of the staphylopine biosynthetic genes (*y2836-y2834*). In a *ΔznuBC* background, mutation of *y2842,* which encodes the periplasmic binding protein for the ABC transporter, did not further affect growth under Zn-deficient conditions.[59] In addition, the second gene, encoding a permease, has undergone a frameshift mutation, making it unlikely to be functional.[114] Finally, *Y. pestis* KIM lacks *cntK* whose protein product converts L-histidine to D-histidine, the first step in staphylopine synthesis.[112, 113] Thus the functionality of this system in *Y. pestis* is uncertain.

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Conclusions

This study has demonstrated that Zn^{2+} acquisition by *Y. pestis* is critical for the progression of bubonic and pneumonic plague. We have provided additional evidence that the Ybt siderophore does not directly bind Zn^{2+} but instead we suggest that a modified Ybt molecule or Ybt plus a second compound (YbtZ) may be the zincophore (Zbt). Although additional Zn^{2+}-binding compounds are encoded or expressed by *Y. pestis,* the Znu and YbtX systems remain the only two proven high-affinity Zn^{2+} importers functional *in vitro* or *in vivo.* Finally, while *ΔzntA* and *ΔzntA zur::kan* mutants had increased Zn sensitivity *in vitro* compared to their $ZntA^+$ Zur^+ parent, the *ΔzntA zur::kan* mutant retained high virulence in mouse models of bubonic and pneumonic plague. This suggests that *Y. pestis* either does not encounter toxic Zn levels during intracellular residence or other Zn efflux systems are used *in vivo* to prevent Zn toxicity,

Supplementary Material

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Acknowledgments

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Significance to Metallomics

This study shows that the ability to acquire zinc during infection is critical for Yersinia pestis to cause bubonic and pneumonic plague. Two different zinc transporters are important for growth in mice. While one system compensates for the loss of the other, loss of both causes a drastic loss of virulence. One system involves the yersiniabactin (Ybt) siderophore (important for iron uptake). While the Ybt sideropohre does not directly bind zinc, our results suggest that Ybt plus a second molecule or a modified Ybt participates in or promotes Zn-binding and is involved in the ability of Y. pestis to obtain zinc.

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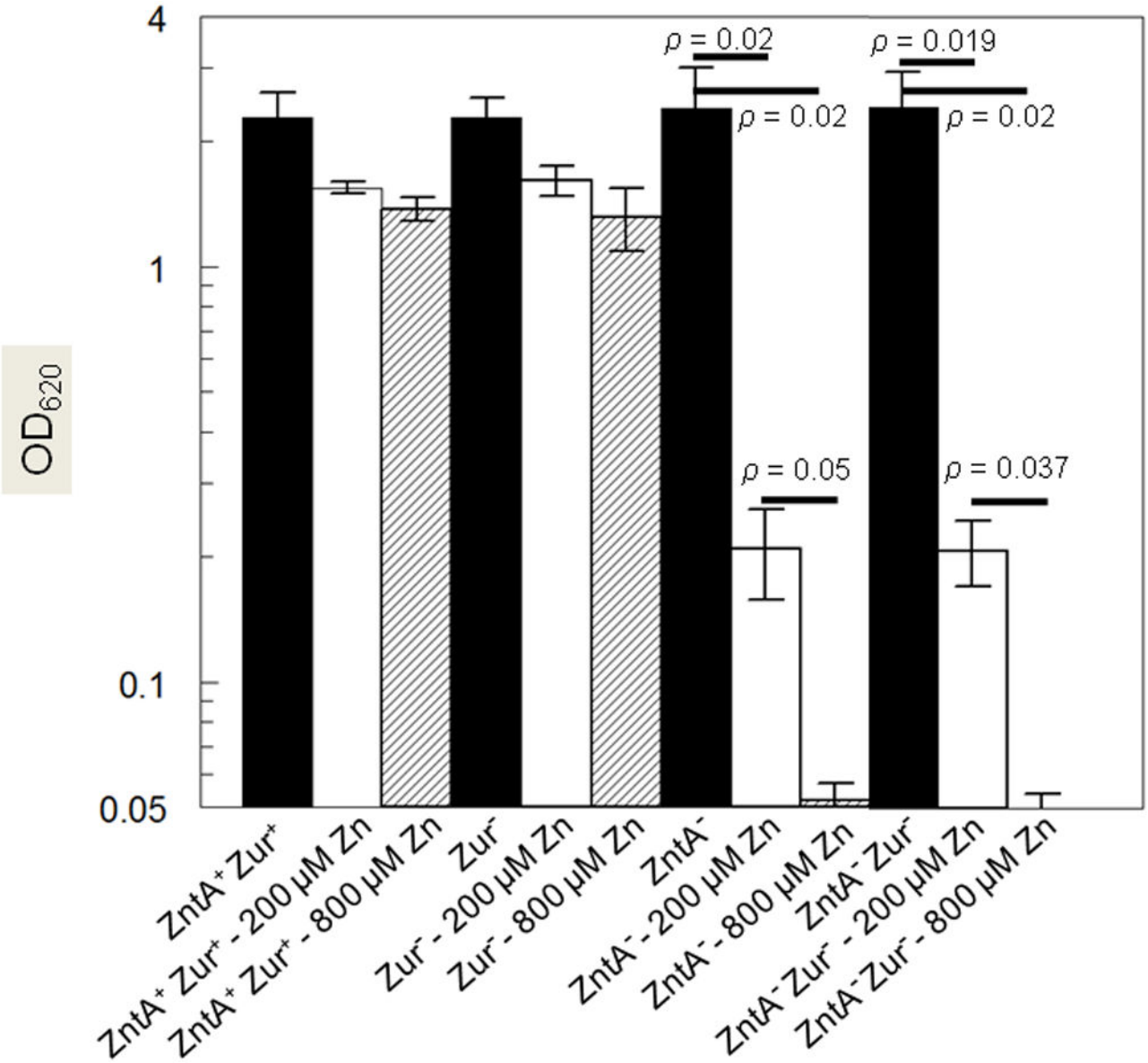

Fig. 1. ZntA but not Zur is required for Y. pestis growth under high Zn^{2+} conditions

Growth rates of *Y. pestis* KIM6+ ($ZntA^+$ Zur^+ parental strain), KIM6-2078+ (Zur^-; *zur::kan*), KIM6-2196.1+ ($ZntA^-$; *ΔzntA*) and KIM6-2196.4+ ($ZntA^-$ Zur^-; *ΔzntA zur::kan*) in HIB-FS without or with supplementation to 200 and 800 μM $ZnC1_2$. Numbers are averages from three independent experiments or cultures. Standard deviations and statistical significances are shown.

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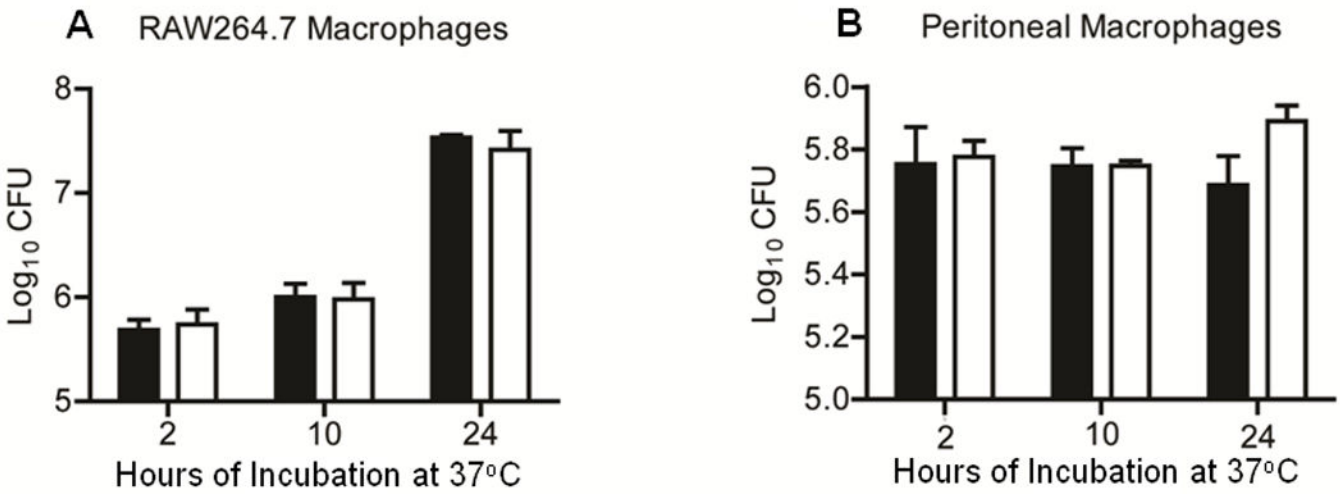

Fig. 2. The Δ*zntA zur::kan* mutant is not attenuated for survival in macrophages

1.0×10^5 (A) RAW274.6 (n=3 samples/time point) or (B) peritoneal macrophages (n=3 samples/time point) were infected with *Y. pestis* KIM6+ ($ZntA^+$ Zur^+) or KIM6-2196.4+ ($ZntA^-$ Zur^-; *ΔzntA zur::kan*) at an MOI of 10. Extracellular bacteria were killed with gentamicin and intracellular bacteria were quantified at 2, 10, and 24 h post-infection. Patterns for RAW274.6 cells (growth) and peritoneal macrophages (survival but not growth) are typical for *Y. pestis*.

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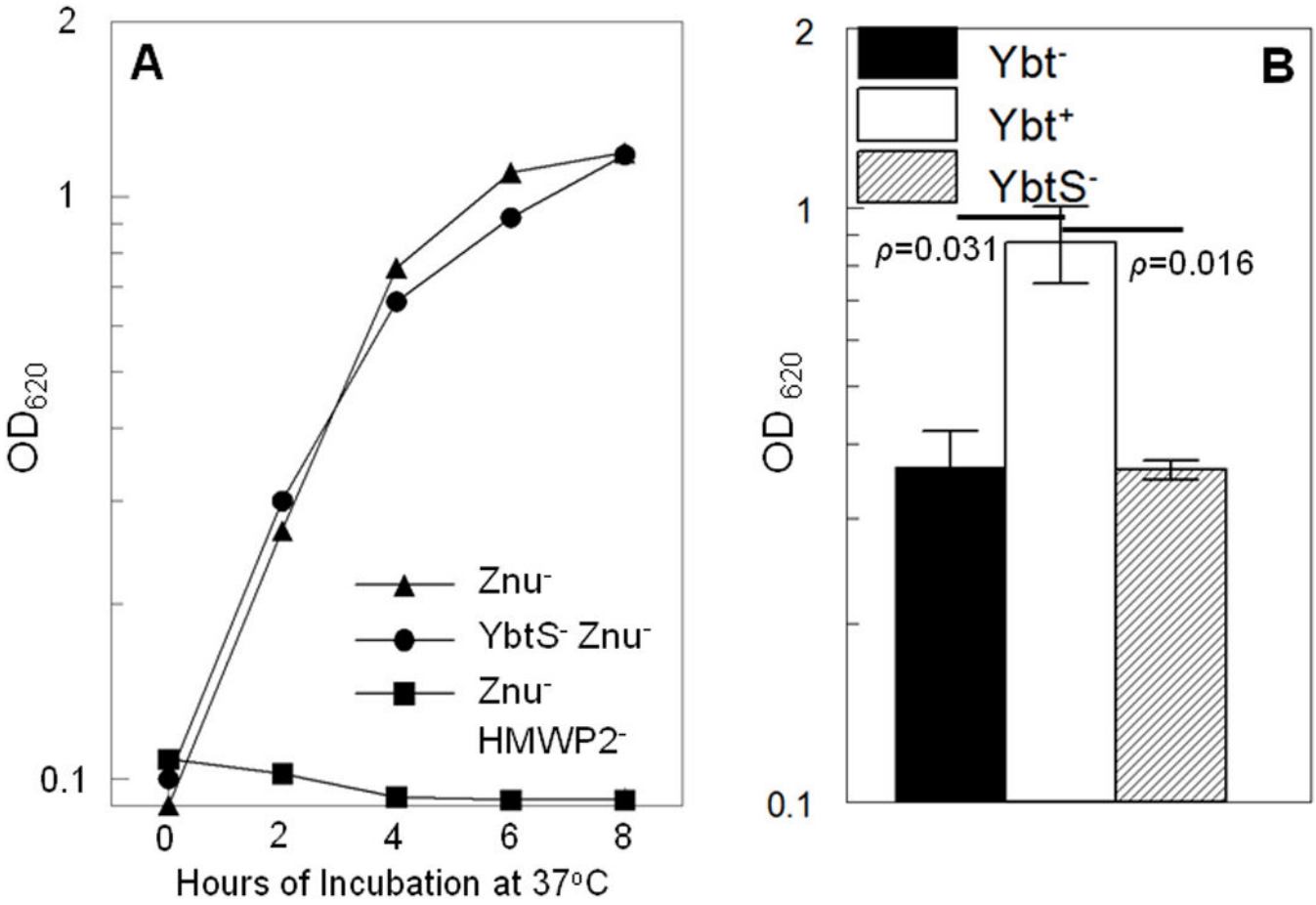

Fig. 3. YbtS is not required for growth of the znu mutant under low Zn^{2+} conditions

Panel A. Growth rates of *Y. pestis* KIM6-2077+ (Znu$^-$; *ΔznuBC*), KIM6-2070.3 (YbtS$^-$ Znu$^-$; *ybtS::kan ΔznuBC*) and KIM6-2077.7 (HMWP2$^-$ Znu$^-$; *irp2::kan ΔznuBC*) in cPMH2 supplemented with 0.6 μM $ZnCl_2$ and 1 μM $FeCl_3$. Panel B. After acclimation to growth at 37°C in cPMH2 supplemented with 0.6 μM $ZnCl_2$ and 1.0 μM $FeCl_3$, cultures of KIM6-2077.18 (HMWP2$^-$ Psn$^-$ Znu$^-$; irp2::kan Δpsn ΔznuBC) were back diluted to an OD_{620} of ~ 0.1 in a 1:1 mixture of the same medium with filtered culture supernatants from KIM6-2046.1 (Ybt$^-$; *irp2::kan*), KIM6+ (Ybt$^+$ parent strain) and KIM6-2070.1 (YbtS$^-$; *ybtS::kan*). Numbers are averages from multiple samples from at least two independent experiments. Standard deviations and statistical significances are shown.

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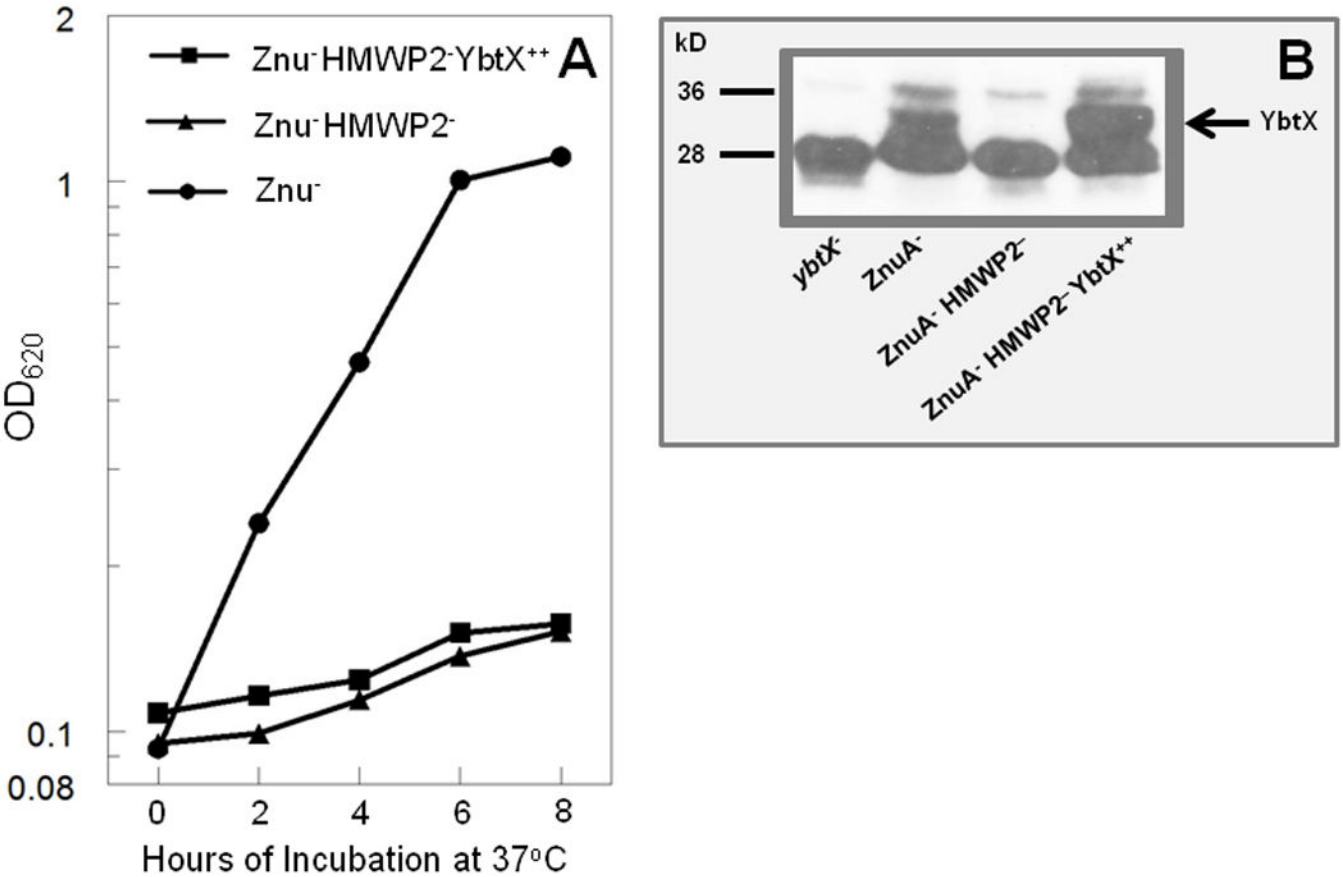

Fig. 4. Overexpression of YbtX does not restore the growth defect of double Znu⁻ HMWP2⁻ mutants under low Zn conditions

Panel A: Growth of *Y. pestis* KIM6-2197(pACYC184)+ (Znu⁻; *ΔznuA*) and KIM6-2197.1 carrying pACYC184 (Znu⁻ HMWP2⁻; *Δirp2 ΔznuA*) or pYbtX-ZP (Znu⁻ HMWP2⁻ YbtX⁺⁺; *Δirp2 ΔznuA ybtX*⁺⁺) in cPMH2 supplemented with 0.6 μM $ZnCl_2$ and 1 μM $FeCl_3$. Over expression of *ybtX*⁺ is driven by the *znuA* promoter. Panel B: Analysis of YbtX expression by Western blot with antiserum against YbtX. KIM6-2067 (YbtX⁻; *ybtS::kan*) is a negative control. The growth curves and Western blot shown are representative of results from two or more independent experiments.

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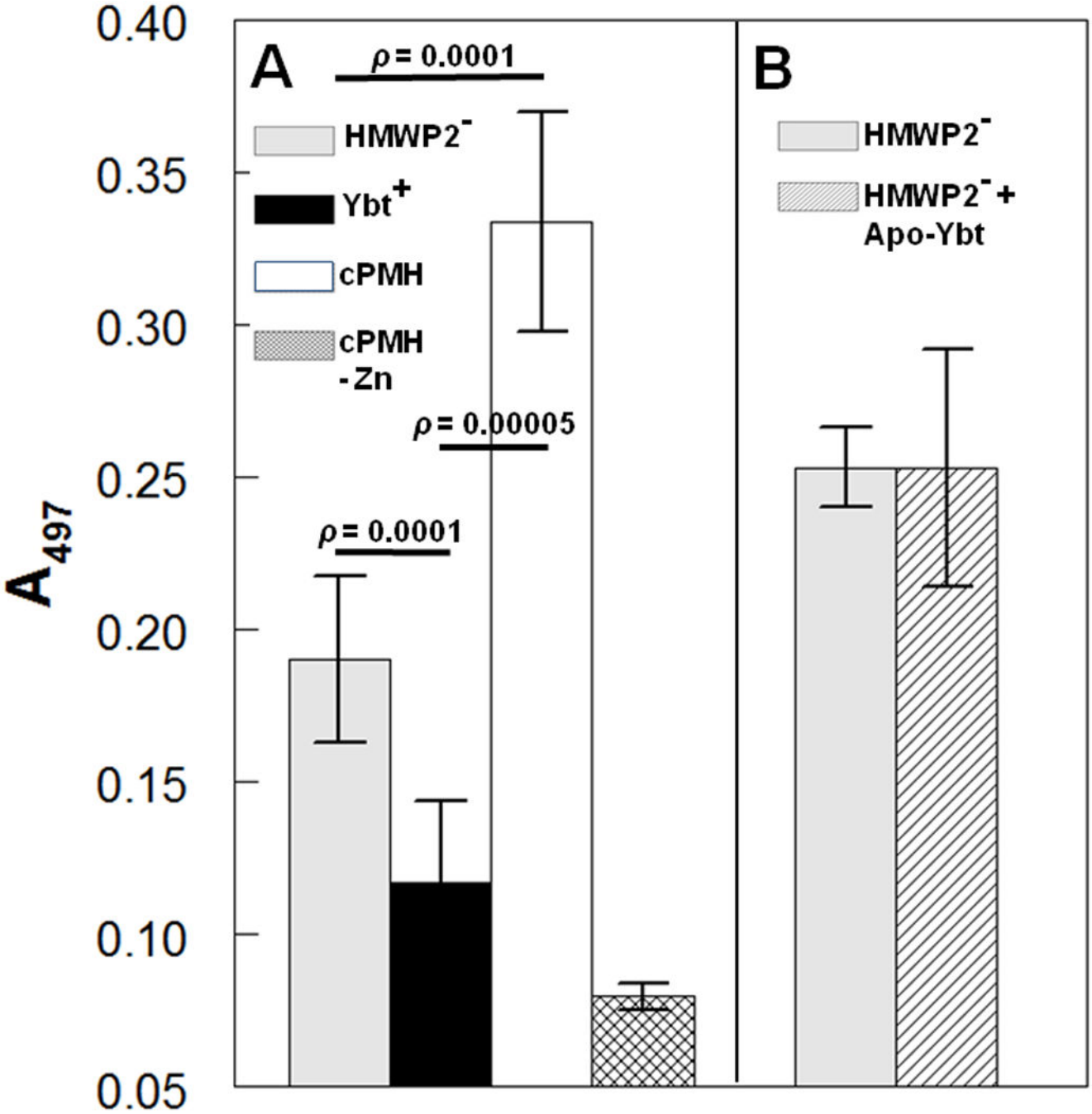

Fig. 5. PAR assay for detection of Zn^{2+}-binding activity in culture supernatants

Filtered supernatants of Ybt producing (KIM6+; Ybt^+) and a non-producing strain (KIM6-2046.1; $HMWP2^-$; *irp2::kan*) (A) grown in cPMH medium for 30 hours at 37°C were incubated with 3 μM (A - supernatant samples) or 5 μM (B – purified apo-Ybt) $ZnCl_2$ in a PAR reaction buffer before PAR was added to 50 μM. cPMH with or without 3 μM $ZnCl_2$ was used as a control. When the PAR assay is performed without added Zn^{2+}, the absorbance change due to Zn^{2+} binding is further reduced as expected. In panel B, apo-Ybt was added to the KIM6-2046.1 supernatant to directly test Zn^{2+} binding by apo-Ybt. Results

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are the average from at least 6 (A) or 2-4 (B) independent samples. Statistical significances are indicated.

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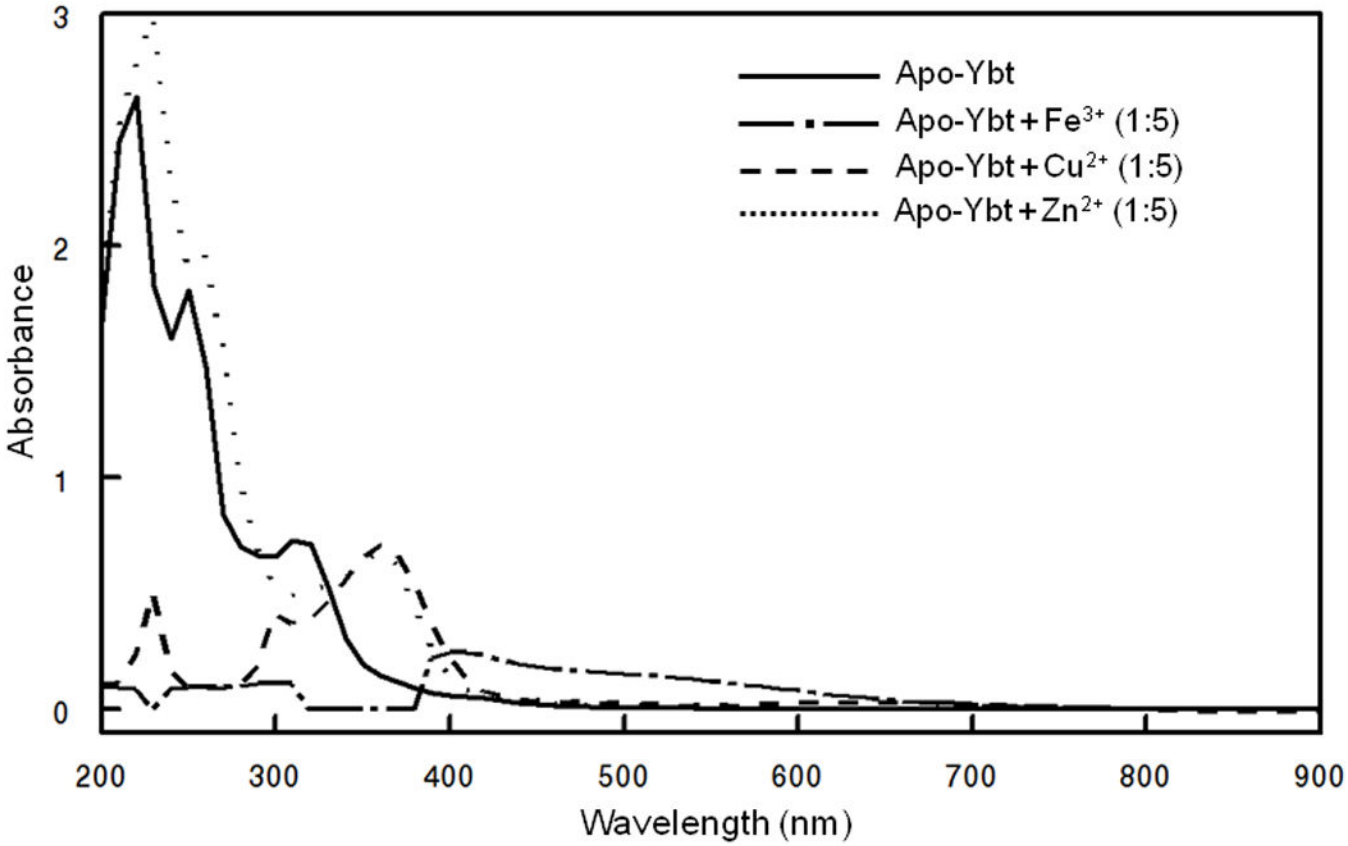

Fig. 6. UV-Vis absorption spectra of apo-Ybt and Ybt + Fe^{3+}, Cu^{2+}, or Zn^{2+}

Metals were added to apo-Ybt at a concentration 5-fold higher than the apo-Ybt concentration two minutes prior to spectroscopic measurements from 200-900 nm.

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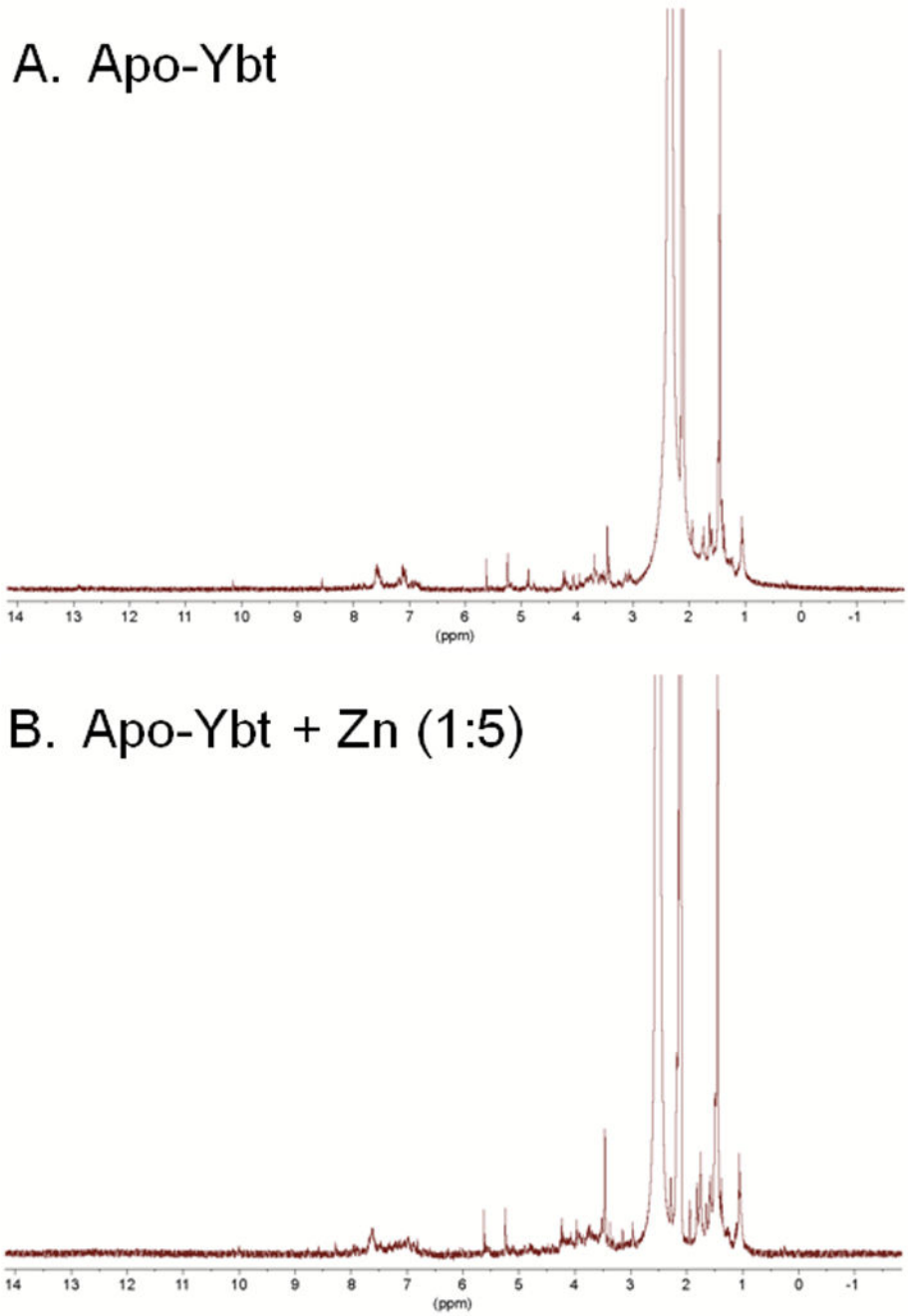

Fig. 7. ^{1}H NMR spectra of apo-Ybt and apo-Ybt + Zn^{2+} (1:5)

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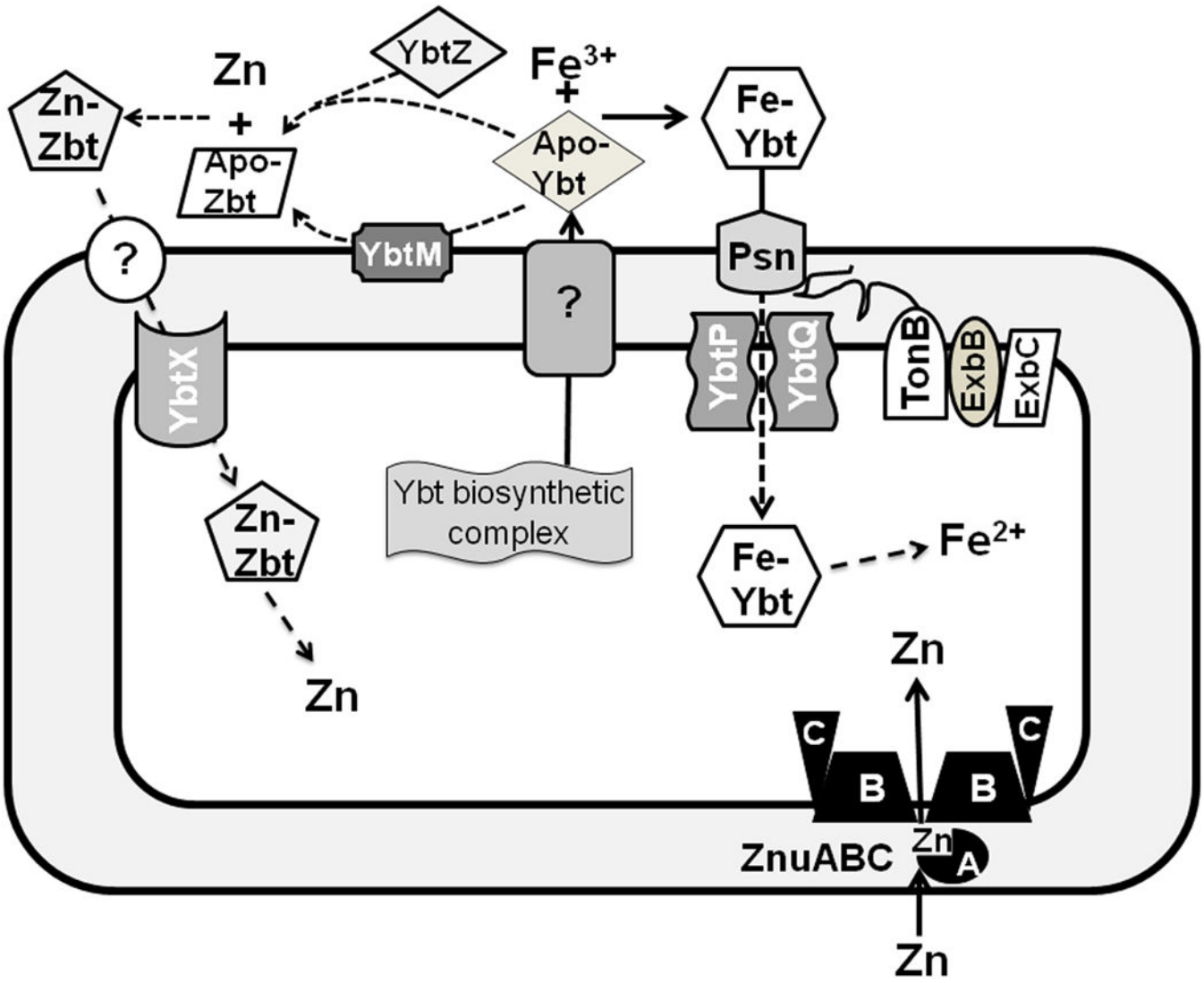

Fig. 8. Model of proposed Zn^{2+} transport in *Y. pestis*

The ZnuABC transporter is typical of other Gram-negative ZnuABC systems. For the Ybt systems, the mechanisms for export of apo-Ybt and removal of Fe from the siderophore have not been established. In addition, entry of the Fe-Ybt chelate into the cell has not been demonstrated (dashed arrows indicate tentative pathways). Use of Fe from Ybt does require the TonB-dependent OM receptor, Psn and the YbtPQ ABC IM transporter (solid arrows indicate proven pathways). Current evidence supports two models for the role of Ybt in Zn^{2+} uptake. A putative surface exposed enzyme (YbtM) modifies Ybt such that it binds Zn^{2+}. Alternatively, exported apo-Ybt may interact with another putative exported compound (YbtZ) to generate a Ybt-YbtZ Zn^{2+} binding complex (tentatively termed apo-Zbt for both models). In the second model, YbtZ is degraded in the absence of apo-Ybt. **If** an OM receptor or porin is required for Zn^{2+} uptake via YbtX, it is not TonB dependent. As for Fe-Ybt, there is no evidence for or against entry of the Zn^{2+}-Zbt complex into the cell.

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Table 1

Virulence of *Y. pestis* strains in mouse models of bubonic, pneumonic and septicemic plague

Strain or mutation[a]	Pneumonic plague LD_{50}[b]	Bubonic plague LD_{50}[b]	Septicemic plague LD_{50}[b]
Parent strain	129 [329 ± 105]	<14 [25 ± 12]	<14 ± 1.4
ΔznuA	715 ± 619	52 ± 58	~11 ± 5
ΔybtX	434 ± 231	176 ± 171[c]	Not tested
ΔybtX ΔznuA	>1.6 × 10^6 ± 2.2 × 10^5 (>1.2 × 10^4-fold)	>8.9 × 10^6 ± 1.6 × 10^5 (>6.4 × 10^5-fold)	Not tested
ybtX^{rp}*Δznu*	976 ±66	32 ± 33 (NS)	Not tested
ybtS::kan ΔznuBC	Not tested	Not tested	5.3 × 10^6 ± 1.2 10^4
ΔzntA zur::kan	1,237 ± 656	17.8 ± 0.4	Not tested

[a]Strains: parent - KIM5(pCD1Ap)+ (Ybt^+ Znu^+); Δ*znuA* – KIM5-2197(pCD1Ap)+; *ΔybtX* – KIM5-2067(pCD1Ap); *ΔybtX ΔznuA* – KIM5-2197.2(pCD1Ap); *ybtX*^{rp}*Δznu* – KIM5-2197.4(pCD1Ap)+ [*ΔybtX* mutation replaced with *ybtX*$^{+}$]; *ybtS::kan ΔznuBC* – KIM5-2070.3(pCD1Ap); *ΔzntA zur::kan* – KIM5-2196.4(pCD1Ap)+

[b]Standard deviations are shown with the fold virulence losses (compared to the parent strain) shown in parentheses, where relevant. Results for the parent strain are from one trial in the pneumonic and bubonic plague models used as a positive control; previous results from multiple trials with the same parent strain[57] are shown in brackets.

[c]One of three trials had a calculated LD_{50} of <13 (lowest dose).