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# Manganese transporters Yfe and MntH are Fur-regulated and important for the virulence of *Yersinia pestis*

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*Yersinia pestis* has a flea-mammal-flea transmission cycle, and is a zoonotic pathogen that causes the systemic diseases bubonic and septicaemic plague in rodents and humans, as well as pneumonic plague in humans and non-human primates. Bubonic and pneumonic plague are quite different diseases that result from different routes of infection. Manganese (Mn) acquisition is critical for the growth and pathogenesis of a number of bacteria. The Yfe/Sit and/or MntH systems are the two prominent Mn transporters in Gram-negative bacteria. Previously we showed that the *Y. pestis* Yfe system transports Fe and Mn. Here we demonstrate that a mutation in *yfe* or *mntH* did not significantly affect *in vitro* aerobic growth under Mn-deficient conditions. A *yfe mntH* double mutant did exhibit a moderate growth defect which was alleviated by supplementation with Mn. No short-term energy-dependent uptake of $^{54}$Mn was observed in this double mutant. Like the *yfeA* promoter, the *mntH* promoter was repressed by both Mn and Fe via Fur. Sequences upstream of the Fur binding sequence in the *yfeA* promoter converted an iron-repressible promoter to one that is also repressed by Mn and Fe. To our knowledge, this is the first report identifying *cis* promoter elements needed to alter cation specificities involved in transcriptional repression. Finally, the *Y. pestis yfe mntH* double mutant had an ~133-fold loss of virulence in a mouse model of bubonic plague but no virulence loss in the pneumonic plague model. This suggests that Mn availability, bacterial Mn requirements or Mn transporters used by *Y. pestis* are different in the lungs (pneumonic plague) compared with systemic disease.

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## INTRODUCTION

The importance of manganese (Mn) for intermediary metabolism, transcriptional regulation and virulence of pathogens has become apparent in recent years (Anderson *et al.*, 2009; Guedon & Helmann, 2003; Jakubovics & Jenkinson, 2001; Kliegman *et al.*, 2006; Ouyang *et al.*, 2009; Papp-Wallace & Maguire, 2006; Schmitt, 2002; Zaharik & Finlay, 2004). The loss of virulence and/or intracellular survival caused by mutations in Mn transport systems has been documented in a number of different pathogens, including *Borrelia burgdorferi*, *Brucella abortus*, *Neisseria gonorrhoeae*, *Porphyromonas gingivalis*, *Salmonella*, various *Streptococcus* species and *Yersinia pseudotuberculosis* (Anderson *et al.*, 2009; Arirachakaran *et al.*, 2007; Berry & Paton, 1996; Boyer *et al.*, 2002; Dintilhac *et al.*, 1997; He *et al.*, 2006; Janulczyk *et al.*, 2003; Kehres *et al.*, 2002a; Lim *et al.*, 2008; Marra *et al.*, 2002; Ouyang *et al.*, 2009; Paik *et al.*, 2003; Smith *et al.*, 2003).

In the course of characterizing iron (Fe) transport systems of *Yersinia pestis*, a zoonotic pathogen that causes the systemic diseases bubonic and septicaemic plague in rodents and humans, as well as pneumonic plague in humans and non-human primates (Inglesby *et al.*, 2000; Perry & Fetherston, 1997), we identified the ABC transporter YfeA-E, which transports Fe and Mn. Deletions or insertions into the *yfeABCD* operon in a strain which does not make the siderophore yersiniabactin caused significant growth inhibition under Fe-chelated conditions and reduced Fe and Mn

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FBS, Fur binding site; IN, intranasal; SBP, substrate binding protein; SC, subcutaneous(ly).

A supplementary figure and a supplementary table are available with the online version of this paper.

 

uptake. In this same background, a Δ*yfeE* mutant had only a modest growth delay due to Fe chelation. The *yfeE* gene is encoded near the *yfeABCD* operon but transcribed from a separate promoter. The *yfeABCD* and *yfeE* promoters both have putative Fur binding sites (FBSs). Although the *yfeA–D* promoter was repressed by Fe and Mn in a Fur-dependent manner, the *yfeE* promoter was unaffected by a surplus of either of these cations. In a slightly attenuated background, a Δ*yfeAB2031.1* mutation caused an ~75-fold loss of virulence in a mouse model of bubonic plague (Bearden *et al.*, 1998; Bearden & Perry, 1999; Perry *et al.*, 2003).

With two exceptions (FeoB2 of *P. gingivalis* and BmtA of *B. burgdorferi*) (Dashper *et al.*, 2005; He *et al.*, 2006; Ouyang *et al.*, 2009), demonstrated Mn transporters fall into two categories: MntH of the NRAMP1 family and the cluster A-1 family of substrate binding proteins (SBPs). Members of the cluster were previously classified as the c9 family of ABC transporters. Some members of the A-1 cluster show specificity for Mn or Zn; others seem to have multiple transition metal substrates (Berntsson *et al.*, 2010; Claverys, 2001; Papp-Wallace & Maguire, 2006). The Yfe/Sit systems, which are members of this cluster, have been shown to acquire Mn and Fe in *Y. pestis*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Shigella flexneri* (Bearden *et al.*, 1998; Bearden & Perry, 1999; Desrosiers *et al.*, 2010; Hazlett *et al.*, 2003; Janakiraman & Slauch, 2000; Janulczyk *et al.*, 1999, 2003; Kehres *et al.*, 2002a; Paik *et al.*, 2003; Runyen-Janecky *et al.*, 2006, 2003; Sabri *et al.*, 2006).

In this study we examine the Mn regulation of the *Y. pestis mntH* and *yfe* promoters as well as the role of these systems in Mn uptake and virulence. Our *in vitro* analyses indicate that Yfe and MntH serve semi-redundant functions in Mn acquisition. Mutation of both systems results in a modest growth inhibition and complete loss of short-term, energy-dependent $^{54}$Mn uptake. Like the *yfeABCD* promoter, the *mntH* promoter is repressed by both Fe and Mn through Fur. Both promoters show similarity to each other in their FBSs and sequences immediately upstream of the FBS. Transfer of a small region of the *yfeA* promoter converted the Fur-regulated *hmuP′* promoter, which is repressed by Fe but not Mn, to a chimeric promoter that is repressed by both cations. In virulence studies, the *yfeAB mntH* double mutant had an ~133-fold loss of virulence in a mouse model of bubonic plague compared with its Yfe$^+$ MntH$^+$ parent. This loss of virulence is greater than would be predicted from our *in vitro* Mn-deficient growth results. Intriguingly, the *yfeAB mntH* mutant was fully virulent in a mouse model of pneumonic plague.

## METHODS

**Bacterial strains and cultivation.** The bacterial strains used in this study are described in Supplementary Table S1. From glycerol stocks (Beesley *et al.*, 1967), Pgm$^+$ and Pgm$^-$ *Y. pestis* strains were streaked onto Congo red (CR) agar (Surgalla & Beesley, 1969) and incubated at 28–30 °C for 2–3 days prior to transfer of a red or white colony to tryptose blood agar base (TBA) slants. Red colonies on CR plates have retained the chromosomal *pgm* locus which encodes numerous genes including the yersiniabactin (Ybt) siderophore-dependent Fe transport system, FetMP (an $Fe^{2+}$ transporter) and the Hms biofilm locus. The *pgm* locus spans 102 kb and has an *in vitro* spontaneous deletion rate of $10^{-5}$. Strains with a 'plus' designation (e.g. KIM6+) have an unmutated *pgm* locus. Strains without a 'plus' designation (e.g. KIM6) either have a mutation within the *pgm* locus or have deleted the entire locus (Brubaker, 1969; Fetherston *et al.*, 2010; Perry *et al.*, 2012). *E. coli* DH5α and DH5α (λ pir) were used to propagate recombinant plasmids.

For growth studies of Mn acquisition, *Y. pestis* cells were harvested from TBA slants after 1–2 days of incubation at 30 °C and grown in a chemically defined medium, PMH2 (pH 7.5), which had been extracted prior to use with Chelex-100 resin (Bio-Rad Laboratories). Correct PIPES and HEPES concentrations should be 50 mM for PMH2 and PMH, respectively, not micromolar concentrations as previously published (Gong *et al.*, 2001; Staggs & Perry, 1991). After Chelex-100 extraction of PMH2, the mean Mn concentration was 0.46 μM (±0.14 μM) (Cornell Nutrient Analysis Laboratory). *Y. pestis* strains were also cultivated in PMH2 supplemented with $MnCl_2$ to various concentrations. All glassware used for Mn-restricted growth studies was soaked overnight in ScotClean (OWL Scientific) to remove contaminating metals and copiously rinsed in deionized water. Unless indicated otherwise, cultures were aerated (200 r.p.m.) with culture volumes of about 10–20 % of flask volume. Growth through two transfers (about six to eight generations) was used to acclimate cells to PMH2 and varying Mn conditions prior to use in all experimental studies. For growth studies aimed at identifying additional Mn transport systems, PMH2 was treated with three times the normal Chelex-100 concentration [15 g (100 ml)$^{-1}$ of 2× medium]. For these studies, EDDA [ethylene-di(*o*-hydroxyphenylacetic acid)] treated to remove contamining iron (Rogers, 1973) was added to a final concentration of 0.5 μM for third-transfer cultures. Growth of all cultures was monitored by determining the $OD_{620}$ with a Genesys 5 spectrophotometer (Spectronic Instruments). Where appropriate, ampicillin (Ap; 50–100 μg ml$^{-1}$), chloramphenicol (Cm; 15–30 μg ml$^{-1}$), kanamycin (Km; 50 μg ml$^{-1}$), spectinomycin (Spc; 25–100 μg ml$^{-1}$), streptomycin (Sm; 50 μg ml$^{-1}$) or tetracycline (Tc; 12.5 μg ml$^{-1}$) was added to media.

**$^{54}$Mn uptake studies.** *Y. pestis* cells were grown aerobically in Chelex-100-treated PMH2 for about five generations prior to use in transport assays. Transport was initiated by the addition of $^{54}MnCl_2$ at a final concentration of 0.4 μCi ml$^{-1}$ (14.8 kBq ml$^{-1}$). Parallel cultures preincubated for 10 min with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were used to demonstrate energy-independent binding of Mn. Transport assay samples (0.5 ml) were withdrawn at various times after the addition of labelled Mn, filtered through 0.22 μm pore-size nitrocellulose membranes (Millipore) and rinsed twice with PMH2 medium containing 20 μM $MnCl_2$, as previously described. Samples, in the absence of scintillation cocktail, were counted in a Cobra II Auto-Gamma counting system (Packard Instruments) with a 15–2000 keV window. Duplicate, unfiltered samples were used to determine the total amount of radioactivity in each culture. The results are expressed as percentage uptake per 0.4 $OD_{620}$ units to compensate for increases in cell density during the course of the assay (Bearden *et al.*, 1998; Bearden & Perry, 1999; Perry *et al.*, 2003).

**Plasmids and DNA techniques.** Plasmids (see Supplementary Table S1) were purified by alkaline lysis and transformed into *E. coli* strains by standard calcium chloride transformation or electroporated into *Y. pestis* cells as previously described (Ausubel *et al.*, 1987; Birnboim & Doly, 1979; Fetherston *et al.*, 1995). DNA restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, Klenow and PCR

amplifications followed manufacturer's specifications. Constructed mutations were confirmed by PCR or restriction enzyme digests. Sequences of PCR amplicons used for cloning genes and promoter regions were confirmed by ACGT, Inc. or Elim Pharmaceuticals. Supplementary Table S1 lists the primers (with their sequences) used in PCRs.

**Construction of *Y. pestis* mutants.** Construction of the Δ*mntH2122* mutation in Δ*pgm* strains KIM6 and KIM6-2031.1 (Δ*yfeAB2031.1*), generating strains KIM6-2122 and KIM6-2122.1, has been reported previously (Hazlett *et al.*, 2003). Here we constructed the same mutation in $Pgm^+$ backgrounds using the suicide vector pΔMntH, generating KIM6-2122+ (Δ*mntH2122*) and KIM6-2122.1+ (Δ*mntH2122* Δ*yfeAB2031.1*). Primers Yp MntH 5′ *Sal*I and Yp MntH 3′ *Spe*I were used to confirm the Δ*mntH2122* mutation in these strains.

For construction of multiple divalent cation transport mutants, we started with *Y. pestis* strain KIM6-2163.5(pWL204) (Δ*yfeABCD2031.4* Δ*feoB2088* Δ*fetMP2163.5*) and used a combination of red recombinase methods (Datsenko & Wanner, 2000; Lathem *et al.*, 2007) and suicide vectors to introduce new mutations. A Δ*efeUOB*::*kan2164.1* mutation was introduced into this strain by the red recombinase system using primers YST-1 and YST-2 to amplify the $Km^r$ cassette in pKD4. The PCR product was electroporated into electrocompetent cells and mutants were selected on TBA plates containing Km. The Δ*efeUOB*::*kan2164.1* mutation was confirmed in several $Km^r$ colonies by PCR with primers YST-3 and YST-4. One mutant was selected and designated KIM6-2163.6(pWL204). The suicide plasmid pΔMntH was introduced into KIM6-2163.6(pWL204) by electroporation and a second cross selected. The introduction of the Δ*mntH2122* mutation was verified by PCR using primers mntH-up and mntH-down and the strain was cured of pWL204. The resulting strain was designated KIM6-2163.7 (Δ*yfeABCD2031.4* Δ*feoB2088* Δ*fetMP2163.5* Δ*efeUOB*::*kan2164.1* Δ*mntH2122*). Plasmid pSkippy was introduced into this strain to remove the *kan* cassette from Δ*efeUOB*::*kan2164.1*, resulting in a Δ*efeUOB2164.1* mutation (KIM6-2163.8). Plasmid pWL204 was reintroduced and primers Y2842-KM1 and Y2842-KM2 were used to generate a Δ*y2482*::*kan2183* strain. The mutation was verified by PCR using primers Y2842-CR and KM-2, and the strain was cured of pWL204 and designated KIM6-2163.11. A *znuBC 2077* mutation was introduced into this strain using the suicide plasmid pSUCZnu3.5; the mutation was confirmed by PCR using primers Znu3.2 and Znu del 1 and the strain was designated KIM6-2163.12.

**Construction of *mntH*::*lacZ* and chimeric *hmu/yfeA*::*lacZ* reporter plasmids.** A 325 bp region immediately upstream of the start codon for *Y. pestis mntH* was amplified from KIM6+ DNA using primers MntH-P1 and MntH-P2 (Supplementary Table S1). The PCR product was digested with *Asc*I and *Asp*718 and cloned into pNEB193. Sequence analysis revealed that the promoter region was intact but that some changes had occurred to the flanking vector sequences during the cloning process. An *Asp*718/*Ecl*136II digest liberated the intact *mntH* promoter (with no errors) from the pNEB193 clone, and this 341 bp fragment was subcloned into the *Asp*718/*Asc*I sites in pEU730 to yield pEUMntH-P (Supplementary Table S1).

Two hybrid promoters were constructed in which the region upstream of the putative FBS in the *hmuP′* promoter was replaced by sequences from the *yfeA* promoter region. Overlapping primer pairs Hmu1up and Hmu1down as well as Hmu2up and Hmu2down were extended in separate PCRs to generate 6 and 15 bp substitutions in the *hmu* promoter, respectively. The products of both reactions were then amplified using primers Hmu/Yfe3 and Hmu/Yfe5. The resulting hybrid promoters were digested with *Asp*718 and *Asc*I, cloned into the corresponding sites of pNEB193 and sequenced. Hybrid promoter regions containing the correct sequence were subsequently cloned into the *Asp*718 and *Asc*I sites in front of *lacZ* in pEU730 to generate pEUhmu/yfe6 and pEUhmu/yfe15.

***β*-Galactosidase assays.** Reporter plasmids with *mntH*::*lacZ* (pEUMnth-P), *hmuP′*::*lacZ* (pHMU44), *yfeA*::*lacZ* (pEUYfeA), *hmu/yfe6*::*lacZ* (pEUhmu/yfe6) or *hmu/yfe15*::*lacZ* (pEUhmu/yfe15) promoter fusions were electroporated into *Y. pestis* KIM6+ ($yfe^+$ $mntH^+$) and/or KIM6-2030+ (*fur*::*kan9*). For KIM6-2030(pEUMnth-P)+, the *fur* mutation was complemented with plasmids expressing *Y. pestis fur* (pFUR1; $fur_{Yp}{}^+$) or *E. coli fur* (pMH15; $fur_{Ec}{}^+$). Cells were acclimated to growth at 37 °C in Chelex-100-treated PMH2 as described above, with Mn and Fe supplementation as indicated. Cells were harvested during early exponential phase. β-Galactosidase activities from whole-cell lysates were measured spectrophotometrically with a Genesys 5 spectrophotometer (Spectronic Instruments) following cleavage of ONPG, and the results are expressed in Miller units (Miller, 1992). Assays used two to six replicate samples and the results are means from two or more independent cultures. For assays using hybrid promoter fusions, independent experiments had absolute β-galactosidase values that varied. Consequently, these results are presented as percentage β-galactosidase activity, with the activity in samples with no Mn or Fe supplementation set to 100 %. These results were calculated from four to six replicate samples from six to eight independent cultures.

**Virulence testing.** Construction and testing of potentially virulent strains were performed in a CDC-approved BSL3 laboratory following Select Agent regulations using procedures approved by the University of Kentucky Institutional Biosafety Committee. *Y. pestis* strains were transformed with pCD1Ap by electroporation (Forman *et al.*, 2007; Gong *et al.*, 2001), plasmid profiles analysed, and transformant phenotypes determined on CR agar (Surgalla & Beesley, 1969) and magnesium oxalate plates (Higuchi & Smith, 1961). After growth at 37 °C in PMH2 with and without $CaCl_2$, culture supernatants were tested for LcrV secretion by Western blotting using polyclonal antisera against histidine-tagged LcrV. Growth at 37 °C without $CaCl_2$ causes growth restriction, expression of the pCD1-encoded type III secretion system, and secretion of LcrV and Yops (Fields *et al.*, 1999; Forman *et al.*, 2007).

Subcutaneous (SC) infection and intranasal (IN) instillation of mice have been previously described (Fetherston *et al.*, 2010). Briefly, *Y. pestis* cells were grown in heart infusion broth (HIB) at 26 °C, resuspended in mouse isotonic PBS (Bearden *et al.*, 1997), and 0.1 ml of 10-fold serially diluted bacterial suspensions was injected SC into groups of four 6- to 8-week-old female Swiss Webster mice (Hsd::ND4). For IN infections, cells were grown at 37 °C in HIB with 4 mM $CaCl_2$ to prevent full induction of Lcr *in vitro* and were similarly diluted in mouse isotonic PBS. Twenty microlitres of the bacterial suspension was administered to the nares (~5 μl aliquots alternating between the two nostrils) of mice sedated with ketamine and xylazine. Administered IN and SC bacterial doses were enumerated by plating serial dilutions on TBA plates containing Ap (50 μg $ml^{-1}$) and colonies were counted after 2 days of incubation at 30 °C (Fetherston *et al.*, 2010). Mice were observed daily for 2 weeks and $LD_{50}$ values were calculated according to the method of Reed & Muench (1938). All animal care and experimental procedures were conducted in accordance with the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals, PHS Policy and the US Government Principles for the Utilization of and Care for Vertebrate Animals in Teaching, Research, and Training, and approved by the University of Kentucky Institutional Animal Care and Use Committee. The University of Kentucky Animal Care Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, Inc.

**Bioinformatics.** The sequenced *Y. pestis* KIM10+ genome (Deng *et al.*, 2002) was used to search for Mn transport and regulatory systems. KIM10+ is a derivative of KIM6+ cured of two virulence plasmids (pCD1 and pPCP1) (Perry *et al.*, 1990).

## RESULTS AND DISCUSSION

### Bioinformatic analysis of potential Mn transporters in *Y. pestis* KIM

We had previously implicated the Yfe ABC transporter in Fe and Mn uptake in *Y. pestis* (Bearden & Perry, 1999; Desrosiers *et al.*, 2010). To identify any additional SBPs in *Y. pestis* KIM that might be involved in Mn transport, we searched the KIM10+ genome with the sequence of MntC, the periplasmic Mn-binding component of an ABC transporter from *N. gonorrhoeae* (Lim *et al.*, 2008) but failed to identify any periplasmic binding proteins (i.e. SBPs) with significant similarity. The *B. burgdorferi* Mn transporter BmtA (Ouyang *et al.*, 2009) does not have an orthologue in the *Y. pestis* KIM10+ genome. The sequence of *P. gingivalis* FeoB2, which transports Mn (Dashper *et al.*, 2005; He *et al.*, 2006), identified only one FeoB, which has a demonstrated role in ferrous iron acquisition (J. D. Fetherston and others, unpublished results; Perry *et al.*, 2007). BLAST analysis did, however, identify Y1500 as an Nramp1-family member with high similarity to *E. coli* MntH. *Y. pestis* MntH is a typical family member with a 409 aa ORF, predicted to encode a 43.6 kDa inner-membrane (IM) protein with 11 transmembrane domains (Deng *et al.*, 2002; TMHMM Server v.20). In addition, a recent study in *Y. pseudotuberculosis* demonstrated that an *mntH* mutant had reduced Mn but not Fe accumulation (Champion *et al.*, 2011). Thus our bioinformatic analyses identified only Yfe and MntH as proven Mn transporters.

### Both Yfe and MntH are involved in Mn acquisition during *in vitro* growth

Strains with a Δ*mntH2122* (KIM6-2122) or a Δ*yfeAB2031.1* (KIM6-2031.1) mutation showed no significant difference from their $Pgm^-$ parent (KIM6) during growth at 30 or 37 °C in the defined medium, PMH2 treated with Chelex-100 (residual Mn concentration ~0.5 μM; Fig. 1). However, a double Δ*yfeAB2031.1* Δ*mntH2122* mutant (KIM6-2122.1) exhibited a moderate growth defect compared with the parent strain, which was more pronounced at 30 than at 37 °C (Fig. 1). Thus, under these growth conditions, the Yfe and MntH systems appear to have redundant Mn import functions. The growth defect of the double mutant was alleviated by supplementation with 10 μM $MnCl_2$. However, the growth of the $Yfe^+$ $MntH^+$ parent was only slightly enhanced by Mn supplementation (Fig. 2). This indicates that the Yfe and MntH systems acquire sufficient Mn from the submicromolar concentration in Chelex-100-treated PMH2 to allow full bacterial growth. The growth defect of the double mutant was also alleviated by carriage of recombinant plasmids encoding the *yfeABCDE* locus (pYFE1.2) or *mntH* (pMntH-Op) (Fig. 2; data not shown).

Since the Yfe system also transports Fe, the growth defect in the *yfe mntH* mutant could result from a combination of reduced abilities to acquire Fe and Mn. However, our studies with the Yfe and Feo $Fe^{2+}$ uptake systems have shown that the *Y. pestis yfe feo* double mutant must be grown under

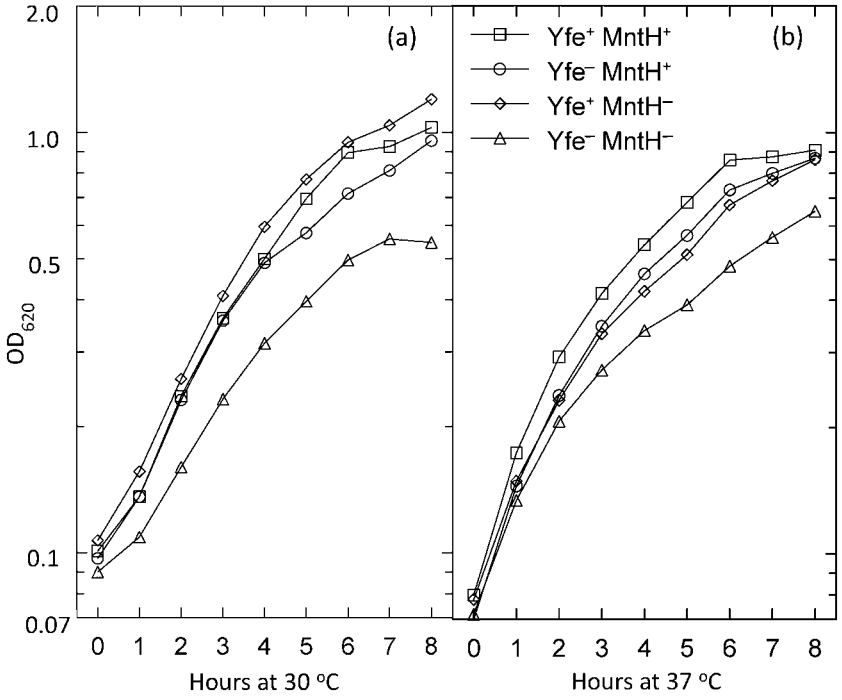


**Fig. 1.** Growth of the *Y. pestis yfeAB mntH* double but not single mutants is reduced in Chelex-100-treated PMH2 at 30 °C (a) and 37 °C (b). Strains: KIM6 ($Yfe^+$ $MntH^+$); KIM6-2031.1 [$Yfe^-$ (Δ*yfeAB2021.1*) $MntH^+$]; KIM6-2122 [$Yfe^+$ $MntH^-$ (Δ*mntH2122*)]; KIM6-2122.1 [$Yfe^-$ (Δ*yfeAB2031.1*) $MntH^-$ (Δ*mntH2122*)]. All strains are Δ*pgm*. The growth curves are from one of two or more independent experiments; all yielded similar results.

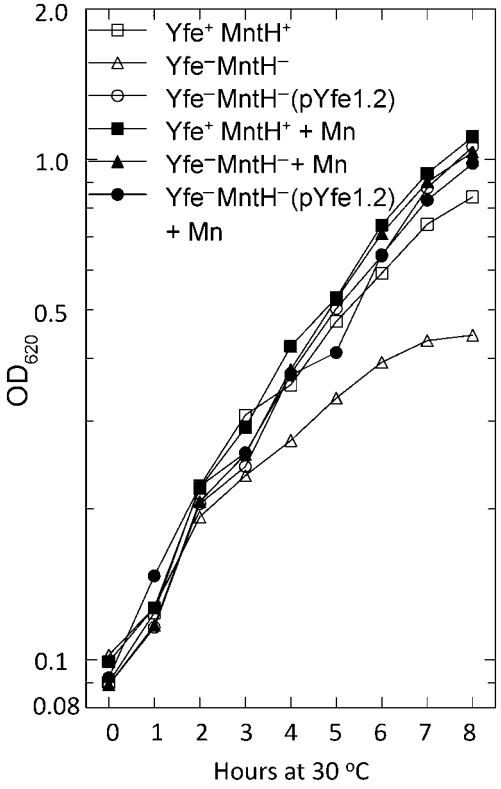


**Fig. 2.** The growth defect of the *Y. pestis yfeAB mntH* double mutant is alleviated by Mn supplementation or by complementation with the *yfeABCDE* locus. All strains are Δ*pgm* and were incubated at 30 °C in Chelex-100-treated PMH2. Where indicated, 10 μM Mn was added. Strains and plasmids: KIM6 ($Yfe^+$ $MntH^+$); KIM6-2122.1 [$Yfe^-$ (Δ*yfeAB2031.1*) $MntH^-$ (Δ*mntH2122*)]; pYfe1.2 encodes the *yfeABCDE* locus. The growth curves are from one of two independent experiments; both yielded similar results.

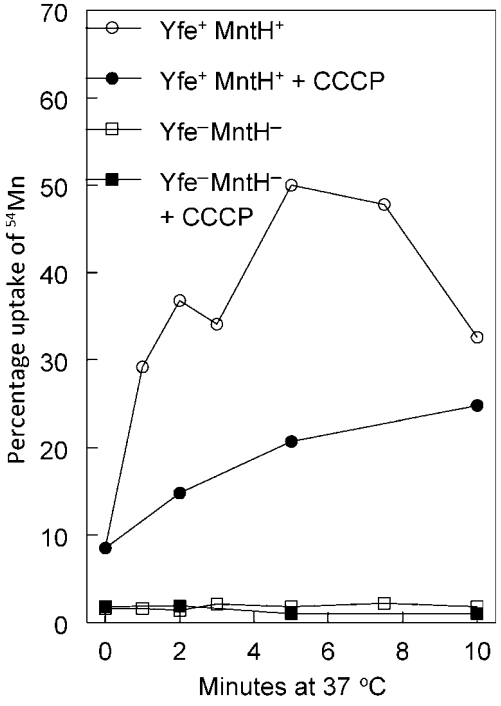


**Fig. 3.** The *Y. pestis yfeAB mntH* double mutant does not actively transport $^{54}Mn$ into the cell. Strains were grown at 37 °C in Chelex-100-treated PMH2, and $^{54}MnCl_2$ was added during early exponential phase to start the Mn uptake assay. For energy-independent binding of $^{54}Mn$ (closed symbols), CCCP was added 10 min prior to radioisotope addition. Strains used were $Pgm^+$: KIM6+ ($Yfe^+$ $MntH^+$) and KIM6-2122.1+ [$Yfe^-$ (Δ*yfeAB2031.1*) $MntH^-$ (Δ*mntH2122*)]. Results are reported as percentage uptake of $^{54}Mn$ per 0.4 $OD_{620}$ unit (cell density). One of two independent experiments with similar results is shown.

microaerobic conditions for this mutant to exhibit a growth defect. In addition, mutations in Yfe and other inorganic Fe uptake systems do not cause a growth defect under Fe-chelated conditions unless the Ybt system is also mutated or absent (Bearden *et al.*, 1998; Bearden & Perry, 1999; J. D. Fetherston and others, unpublished results; Kirillina *et al.*, 2006; Perry *et al.*, 2003, 2007). In contrast, Supplementary Fig. S1 shows that the *Y. pestis yfeAB mntH* double mutant exhibited a growth defect even when the Ybt Fe transport system encoded within the *pgm* locus is present. As a whole, these results indicate that the modest growth defect of the *Y. pestis yfeAB mntH* mutant is due to decreased Mn uptake when grown in trace concentrations of the metal and is not the result of reduced Fe acquisition.

To confirm the loss of Mn acquisition directly, we performed $^{54}Mn$ uptake studies. Previously we demonstrated that a *yfe* mutation reduced energy-dependent $^{54}Mn$ uptake by ~50 % over a 20 min period (Bearden *et al.*, 1998; Bearden & Perry, 1999; Perry *et al.*, 2003). Fig. 3 shows that the *yfe mntH* mutant exhibits no energy-dependent uptake over a 10 min period in PMH2. In contrast the $Yfe^+$ $MntH^+$ parent strain accumulated 40–50 % of the extracellular Mn over the same time period (with no additional significant energy-dependent uptake over 40 min). Thus, despite the moderate growth defect in PMH2, the *yfe mntH* double mutant has no active uptake of Mn at a submicromolar concentration, at least over relatively short time periods. Note that in cells depleted of energy by exposure to CCCP, there is a high level of energy-independent binding of Mn by the parent but not the mutant strain. Previous studies showed that the *yfe* mutant also exhibits low-level energy-independent Mn binding that is similar to that of the *yfe mntH* double mutant (Fig. 3) (Bearden *et al.*, 1998; Bearden & Perry, 1999; Perry *et al.*, 2003). Thus, we believe that the cell-associated Mn observed in the CCCP-treated $Yfe^+$ $MntH^+$ parent is due to YfeA binding Mn in the periplasm of cells expressing this SBP.

The modest *in vitro* growth defect and intermediate loss of virulence in the bubonic plague model (see below) by the double Δ*yfeAB* Δ*mntH* mutant indicate that *Y. pestis* might have additional transporter(s) capable of Mn acquisition

from Mn-deficient environments *in vivo* and *in vitro*. Bioinformatic analyses identified only Yfe and MntH as potential Mn transporters. However, sequence similarities of transporters do not always correctly predict their metal specificities (Lim *et al.*, 2008; Rhodes *et al.*, 2005). Since the Yfe system transports both $Fe^{2+}$ and Mn, we first focused on proven and putative $Fe^{2+}$ transporters: FeoABC, EfeUOB and FetMP (Cao *et al.*, 2007; J. D. Fetherston and others, unpublished results; Große *et al.*, 2006; Koch *et al.*, 2011; Rajasekaran *et al.*, 2010). The growth of a quintuplet mutant, KIM6-2163.7, containing Δ*yfeABCD2031.4* Δ*mntH2122* Δ*feoB2088* Δ*fetMP2163.5* and Δ*efeUOB*::*kan2164.1* mutations, in the Chelex-100-treated PMH2 medium showed a defect similar to that of the double Δ*yfeABCD* Δ*mntH* mutant (data not shown). Finally, we tested whether the ZnuABC Zn transporter or an SBP that is a member of the TroA-like superfamily (Y2842) might contribute to trace Mn acquisition. Construction and testing of a septuplet mutant (KIM6-2163.12) failed to show a growth defect more severe than that of the *yfe mntH* mutant. However, this mutant did show a growth response when the Chelex-100-treated PMH2 medium was supplemented with 1 μM Mn (data not shown). This suggests that a high-affinity Mn uptake system is functioning *in vitro* in *Y. pestis*, and that the unidentified system is not a Feo, Fet, Efe, Znu or Y2842 transporter. Alternatively, it is possible that *Y. pestis* has relatively few requirements for Mn and that the modest *in vitro* growth defect and *in vivo* phenotypes of the *yfe mntH* mutant reflect this.

The *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* AfeABCD system is a member of the Yfe/Sit family. An *A. actinomycetemcomitans afe* mutant is defective for Fe acquisition, and the cloned *afe* locus restores growth to a *Y. pestis yfe feo* mutant under Fe-chelated microaerobic conditions and to an *E. coli* mutant during aerobic growth. However, the *yfe mntH* double mutant is not complemented by the Afe system (Perry *et al.*, 2012; Rhodes *et al.*, 2005). Thus, it is likely that the *A. actinomycetemcomitans* AfeABCD system transports $Fe^{2+}$ but not Mn, currently making it a unique member of the Yfe/Sit ABC transporter family. The reason for the inability of the Afe system to transport Mn remains to be determined.

## Transcription from the *mntH* promoter is repressed by Mn or Fe via Fur

Mn and Fe repression of *mntH* and *sit* (*yfe*) has been demonstrated in *E. coli*, *Shigella flexneri* and *S.* Typhimurium, where these two promoters are primarily repressed by Fe via Fur and by Mn through MntR. In *S.* Typhimurium, it has been determined that both transcriptional regulators are capable of some repression of both promoters in response to the reciprocal cation. In all three of these organisms, *mntH* transcription is induced by exposure to $H_2O_2$ through OxyR. In contrast, expression of *mntH* in *Brucella abortus* is repressed by Mn but not by Fe (Anderson *et al.*, 2009; Ikeda *et al.*, 2005; Kehres *et al.*, 2002b; Patzer & Hantke, 2001; Runyen-Janecky *et al.*, 2006). Previously we demonstrated that Mn and Fe repression of the *yfeA* promoter is Fur-dependent (Bearden *et al.*, 1998; Perry *et al.*, 2003). A search of the *Y. pestis* KIM10+ genome failed to identify a homologue of the *E. coli* Mn transcriptional repressor MntR or Mn-responsive members (Mur) of the Fur superfamily in α-proteobacteria. Thus, Mn regulation in *Y. pestis* may rely upon Fur alone.

We tested regulation of the *mntH* promoter using a promoter fusion to *lacZ*. At 30 °C, transcription from this promoter was repressed by both Fe (10-fold) and Mn (2.7-fold), and this pattern of regulation (Fig. 4) was similar to that previously observed for the *yfeA* promoter. Similar to the *yfeA* promoter (Bearden *et al.*, 1998; Perry *et al.*, 2007), repression of the *mntH* promoter by Mn was Fur-dependent. Curiously, a small degree of Fe repression of the *mntH* promoter occurred in the Fur mutant (Fig. 4).

To determine whether Mn repression was specific to *Y. pestis* Fur, we transformed a *Y. pestis fur* mutant carrying the *mntH* reporter with recombinant plasmids expressing $Fur_{Yp}$ or $Fur_{Ec}$. Fig. 5 shows that both *Y. pestis* Fur and the *E. coli* Fur restored transcriptional repression by Mn and Fe on the *mntH*::*lacZ* reporter. Thus the ability to repress transcription with excess Mn may be a general property of

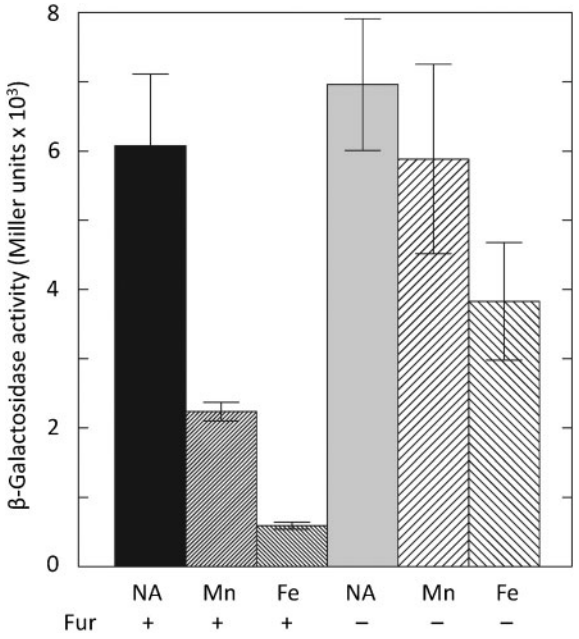


**Fig. 4.** Fur-dependent transcriptional regulation of the *mntH* promoter by Fe and Mn. *Y. pestis* KIM6 (Δ*pgm* $Fur^+$) (bars 1–3 from left) or KIM6-2030 (Δ*pgm fur9*::*kan*) cells (bars 4–6) carrying pEUMntH-P (*mntH*::*lacZ*) were grown in Chelex-100-treated PMH2 at 30 °C with no additions (NA), 10 μM $MnCl_2$ (Mn) or 10 μM $FeCl_3$ (Fe). The values are averages of replicate samples from two or more independent experiments. Error bars, SD.

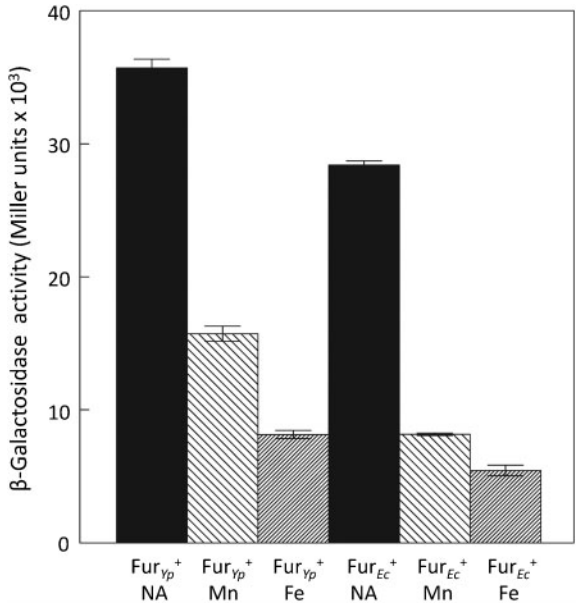


**Fig. 5.** *E. coli* Fur causes Mn-dependent repression of transcription from the *mntH* promoter. *Y. pestis* KIM6-2030 (Δ*pgm fur9*::*kan*) cells carrying pEUMntH-P (*mntH*::*lacZ*) and pFUR1 ($fur_{Yp}^{+}$) or pMH15 ($fur_{Ec}^{+}$) were grown in Chelex-100-treated PMH2 at 37 °C with no additions (NA), 10 μM $MnCl_2$ (Mn) or 10 μM $FeCl_3$ (Fe). The values are averages of replicate samples from two or more independent experiments. Error bars, SD.

Fur proteins. When *fur* is encoded on the *Y. pestis* chromosome, the *mntH* reporter is repressed to a greater extent by Fe compared with Mn (Fig. 4). The approximately equivalent repression by both metals when $fur_{Ec}$ and $fur_{Yp}$ are encoded on plasmids may be due to the increased number of *fur* genes. However, the reason for increased expression under Fe- and Mn-deficient conditions with increased copies of *fur* is unknown (Fig. 5).

While *yfeA* and *mntH* promoters are transcriptionally repressed by excess Mn via Fur, other *Y. pestis* Fe-repressible, Fur-regulated promoters such as those for *fetMP*, *efeUOB* and *yiuABC* are not affected by Mn supplementation (Perry *et al.*, 2012). The Hmu system, an ABC transporter for haemin uptake, is another example of a Fur-regulated operon repressed by Fe but not by Mn. An *hmuP′*::*lacZ* promoter fusion construct (pHMU44) in the same vector as the *yfeA* and *mntH* reporters (pEU730; Supplementary Table S1) showed transcriptional repression after growth at 37 °C in Chelex-100-treated PMH2 containing 10 μM Fe (~16-fold) but not with 10 μM Mn (Fig. 6a). In contrast, our *yfeA*::*lacZ* reporter displayed approximately seven- and 1.8-fold transcriptional repression by Fe and Mn, respectively. Comparing the *yfeA* and *mntH* promoter regions, we noted nucleotide similarities upstream of the FBSs in both promoters (Fig. 7). To determine whether these sequences were involved in Mn-repressible transcription, we constructed two hybrid promoters by replacing 15 or 6 nt of the *hmuP′* promoter region with nucleotides from the *yfeA* promoter region while maintaining the spacing of putative −35, −10 and FBS promoter elements (Fig. 7). Finally, the hybrid promoters were fused to *lacZ* in pEU730. For unknown reasons the activity of the *hms/yfe6* and the native *hmuP* promoter fusions was about threefold higher than that of the *hmu/yfe15* reporter. The hybrid containing 6 nt from *yfeA* (*hmu/yfe6*) was repressed ~1.4-fold by Mn and ~45-fold by Fe. In contrast, the *hmu/yfeA* hybrid promoter with a 15 nt replacement from *yfeA* (*hmu/yfe15*) showed a 2.2-fold transcriptional repression by Mn and a similar repression by Fe (~40-fold) (Fig. 6b). Thus, transcriptional repression by Mn in *Y. pestis* involves Fur and a short nucleotide sequence (>6 and ⩽15 nt) upstream of the putative FBS of *yfeA*. To our knowledge, this study is the first report identifying *cis* promoter elements needed to alter cation specificities involved in transcriptional repression.

Mn regulation by Fur is not unique to *Y. pestis*. Mn repression via Fur has been demonstrated in *E. coli* for the aerobactin locus and for the *fhuF* gene using *lacZ* fusions. The aerobactin locus is fully repressed by 10 μM Mn, while only non-physiological 1 mM Mn was tested for the *fhuF* gene. In contrast, *E. coli sodA* (encoding manganese superoxide dismutase; MnSOD) is repressed by Fur in response to Fe but not to Mn (Bagg & Neilands, 1987; Hantke, 1987; Privalle & Fridovich, 1993). The basis for differential cation regulation of these promoters was not explored. Given Mn repression of the *E. coli iuc* promoter, it is curious that we did not detect Mn repression of the *Y. pestis iuc* promoter (Perry *et al.*, 2012).

## Yfe and MntH are important for the pathogenesis of *Y. pestis* in bubonic but not pneumonic plague models

Mammalian hosts withhold Mn as a component of innate immunity. Mn levels in human blood and plasma have been measured at 0.2–0.3 and 0.04–0.05 μM, respectively; in rat tissues, Mn concentrations from 0.4 to 1.7 μg (g wet tissue weight)$^{-1}$ have been reported. More recently, Mn levels of 0.42 and 1.14 μM in the blood and lungs of mice have been reported. Often, these measurements do not differentiate between free and bound forms of the metal, so the bioavailability of Mn in various organs is uncertain. It has been demonstrated that Mn is bound by apoferritin (*in vitro*) as well as lactoferrin and transferrin (*in vitro* and *in vivo*), and that there are different receptors for Mn-transferrin and Zn-transferrin, at least on mouse mammary gland cells. In addition, calprotectin, a protein shown to be produced by neutrophils in tissue abscesses caused by *Staphylococcus aureus*, chelates Mn and Zn, thereby inhibiting proliferation of the bacterium (Aschner & Aschner, 2005; Aschner & Gannon, 1994; Critchfield & Keen, 1992; Corbin *et al.*, 2008; Davidsson *et al.*, 1989; Kehl-Fie & Skaar, 2010; Lönnerdal *et al.*, 1985; Macara *et al.*, 1973; McDevitt *et al.*, 2011; Moutafchiev *et al.*, 1998; Papavasiliou & Cotzias, 1961; Papp-Wallace & Maguire,

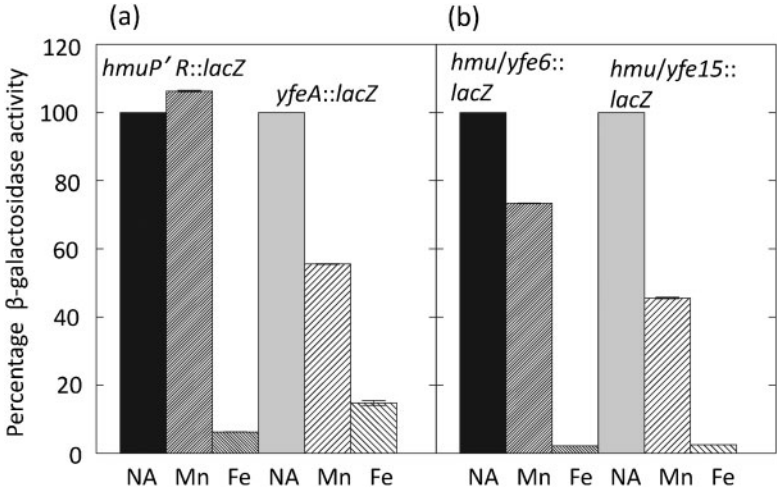


**Fig. 6.** Fifteen nucleotides from the *yfeA* promoter converts *hmuP′* to a Mn-repressible promoter. *Y. pestis* KIM6+ cells carrying pHMU44 (*hmuP′R::lacZ*), pEUYfeA (*yfeA::lacZ*) (a), pEUHmu/Yfe6 (*hmu/yfe6::lacZ*) or pEUHmu/Yfe15 (*hmu/yfe15::lacZ*) (b) were grown in Chelex-100-treated PMH2 at 37 °C with no additions (NA), 10 μM $MnCl_2$ (Mn) or 10 μM $FeCl_3$ (Fe). The two hybrid promoters have 6 and 15 nt from *yfeA* replacing nucleotides in the *hmuP′R* promoter. Due to fluctuations in $\beta$-galactosidase activities among eight independent experiments with four to six replicate samples from each experiment, values are presented as means of percentage activity, with the activities of cells grown without Mn or Fe supplementation set at 100 %. Error bars (most not visible), SD.

2006; Rehnberg *et al.*, 1980; Zaharik & Finlay, 2004). Finally, mutations in bacterial Mn transporters cause a loss of virulence in a number of pathogens. Indeed, MntH and/or the Yfe/Sit family play a key role in *in vivo* Mn acquisition in some pathogens, such as *Brucella abortus*, *S.* Typhimurium and *Y. pseudotuberculosis* (Anderson *et al.*, 2009; Arirachakaran *et al.*, 2007; Berry & Paton, 1996; Boyer *et al.*, 2002; Champion *et al.*, 2011; Dintilhac *et al.*, 1997; He *et al.*, 2006; Janulczyk *et al.*, 2003; Kehres *et al.*, 2002a; Lim *et al.*, 2008; Marra *et al.*, 2002; Ouyang *et al.*, 2009; Paik *et al.*, 2003; Sabri *et al.*, 2008; Smith *et al.*, 2003; Zaharik *et al.*, 2004). The *Y. pestis* strains used in our *in vitro* growth and transcriptional regulation studies are completely avirulent because they lack virulence plasmid pCD1 (Perry & Fetherston, 1997); therefore, we electroporated recombinant plasmid pCD1Ap into the relevant $Pgm^+$ strains under BSL3 conditions to determine the role of Mn transporters in *Y. pestis* virulence. As described in Methods, we used these reconstituted strains in SC and IN instillation infections of Swiss Webster mice as bubonic and pneumonic plague models, respectively.

For bubonic plague, a *yfeAB* mutation caused a reproducible approximately ninefold loss of virulence compared with the parent strain; however, probit analysis indicates that this difference was not significant ($P$=0.55) given the number of animals used. An *mntH* mutant had an $LD_{50}$ similar to that of the parent strain (Table 1). However, the *mntH* mutant did display a 3 day delay in reaching the 50 % end point at an infectious dose about six- to eightfold higher than the calculated $LD_{50}$ (~23 cells) compared with the parent strain. With infectious doses of ~25 cells, more than 60 % of animals survived to day 14; a similar dose (20 cells) of the parent strain was lethal to ~40 % of the mice by day 8 post-infection (Fig. 8). The *yfeAB mntH* double mutant had an ~133-fold loss of virulence in this model ($P$=0.0001) compared with the parent strain (Table 1). This virulence loss is greater than would have been predicted from our *in vitro* Mn-deficient growth results. Consequently, a defect in Mn acquisition does play a significant role in virulence in this mouse model of bubonic plague.

In contrast, the *yfe* single and *yfe mntH* double mutants, via IN instillation (pneumonic plague model), were fully virulent by $LD_{50}$ and time-to-death analyses (Table 1; data not shown). It is unlikely that the lung is a Mn-replete environment since Mn levels have been measured at 1 μM


**Fig. 7.** Sequence comparison of the *hmuP′*, *yfeA* and two hybrid promoter regions. FBSs are in italic type and boxed, while −35 and −10 regions are underlined. The 15 nt from *yfeA* used to replace 15 nt from *hmuP′R* are in bold, lower-case type inside a dashed box in both promoter sequences. For *hmu*/*yfe6*, the 6 nt matching those in *yfeA* are also in bold, lower-case type inside a dashed box. For *mntH*, the corresponding 15 nt are in bold, lower-case type; nucleotides identical to those in *yfeA* are underlined.

**Table 1.** *Y. pestis* $LD_{50}$ values and comparative virulence losses in mouse models of pneumonic and bubonic plague

$LD_{50}$ values ± SD are reported. Virulence loss is compared with the parent/wild-type strain. In the pneumonic plague model, none of the mutations caused a significant loss of virulence as measured by $LD_{50}$. Strains: KIM5(pCD1Ap)+, wild-type; KIM5-2031.12(pCD1Ap)+, Δ*yfeAB2031.1*; KIM5-2122(pCD1Ap)+, Δ*mntH2122*; KIM5-2122.1 (pCD1Ap)+, Δ*yfeAB2031.1* Δ*mntH2122*.

| Strain or mutation | Pneumonic plague | Bubonic plague | |
|---|---|---|---|
| | $LD_{50}$ | $LD_{50}$ | Virulence loss |
| Wild-type | 329 ± 105 | 23 ± 14 | – |
| Δ*yfeAB* | 139 ± 142 | 205 ± 149 | 8.9-fold |
| Δ*mntH* | Not tested | 36 ± 33 | Not significant |
| Δ*yfeAB* Δ*mntH* | 142 ± 4 | 3068 ± 187 | 133-fold |

to submicromolar concentrations in the lung or the sputum of healthy humans. Indeed, an *S. pneumoniae psa* Mn transporter mutant was unable to colonize mouse lungs after IN instillation (Gray *et al.*, 2010; McDevitt *et al.*, 2011). Microarray analysis of *Y. pestis* RNA from the lungs of infected mice showed increased expression of *yfe* but not *mntH* compared to *in vitro* growth. The enhanced expression of *yfe* in the lung was confirmed by quantitative RT-PCR. Thus, the full virulence of the *yfe mntH* double mutant in the pneumonic plague model and the intermediate loss of virulence in the bubonic plague model could be due to: (1) the ability of unidentified Mn transporter(s) to provide sufficient Mn in different organ systems; (2) Mn playing a minor role in *Y. pestis* metabolism and regulation; (3) a shift to metabolic pathways that do not require Mn-dependent enzymes; or (4) a combination of these possibilities.

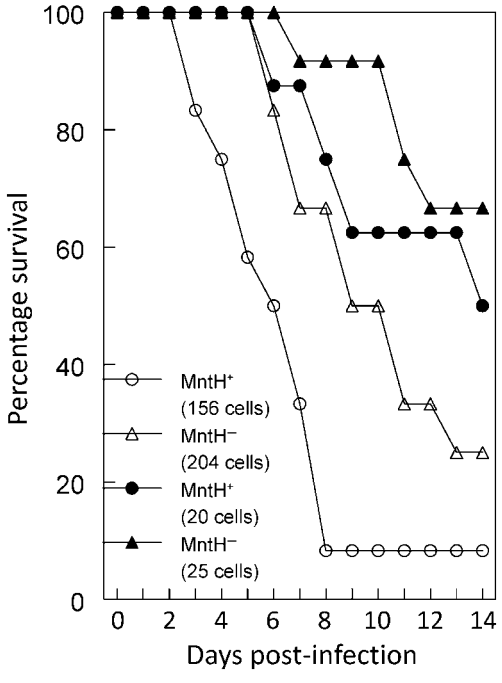


**Fig. 8.** Survival analysis of mice following SC infection with $MntH^+$ or $MntH^-$ *Y. pestis* strains. Low (~1 $LD_{50}$) and mid-range (~6–8× $LD_{50}$) doses (cell dose numbers are shown in parentheses in the figure) were used to infect mice. Two or three independent studies with a total of eight to 12 animals were used in the analysis.

It is intriguing that a *Y. pestis yfe feo* $Fe^{2+}$ uptake mutant showed similar results: significant loss of virulence via SC infection but not by IN infection (J. D. Fetherston and others, unpublished results). These results reinforce our previous conclusion that the importance of Fe transport systems depends upon the organ system in which the bacterium is growing and possibly extends this observation to include Mn transporters.

### Divalent cation homeostasis in *Y. pestis*

Divalent cation homeostasis likely plays a critical role in normal bacterial growth and metabolism, especially in pathogens where the host restricts access to Fe, Mn and Zn. Enzymes with metal cofactors, transport systems, and even transcriptional regulators do not completely discriminate among the relevant divalent transition metal cations. Mn can likely substitute for Fe in some non-redox enzymes, and Zn forms complexes with Mn and Fe metalloproteins (e.g. Fur and MntR appear to have at least limited responses to Mn and Fe, respectively, in some bacteria). However, insertion of an incorrect metal in other proteins may negate their enzymic activity or function (Anjem *et al.*, 2009; Bagg & Neilands, 1987; Fraústo da Silva & Williams, 2001; Hantke, 1987; Ikeda *et al.*, 2005; Privalle & Fridovich, 1993; Tottey *et al.*, 2008). Thus the *in vitro* and *in vivo* phenotypes caused by mutations in *Y. pestis* $Fe^{2+}$, Mn and Zn transporters could result from a combination of starvation for the relevant cation along with insertion of inappropriate cations into proteins.

Fe, Mn and Zn repression of some metabolic enzymes and various cation transporters, as well as the cation specificities of these transporters and their transcriptional regulators, are all likely important for cation homeostasis in *Y. pestis* and other bacteria. In *Y. pestis*, expression of *feoABC* under aerobic conditions in the presence of Fe, the ability of YfeA to bind Zn in the periplasm but not transport it into the cell, as well as dual Mn and Fe repression of *mntH* and *yfe*, may contribute to this homeostasis in different mammalian organ systems and perhaps in the flea (Bearden *et al.*, 1998; Desrosiers *et al.*, 2010; J. D. Fetherston and others, unpublished results; Perry *et al.*, 2003, 2007).

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