MntP and YiiP Contribute to Manganese Efflux in Salmonella enterica Serovar Typhimurium under Conditions of Manganese Overload and Nitrosative Stress

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ABSTRACT The divalent transition metal cation manganese is important for protein function, particularly under conditions of iron limitation, nitrosative stress, and oxidative stress, but can mediate substantial toxicity in excess. Salmonella enterica serovar Typhimurium possesses multiple manganese importers, but the pathways for manganese efflux remain poorly defined. The S. Typhimurium ATCC 14028s genome was analyzed for putative manganese export pathways, which identified a previously uncharacterized homologue of the Escherichia coli manganese exporter mntP, stm1834, and two cation diffusion facilitator family transporters, zitB (stm0758) and yiiP (stm4061). Manganese acquisition by S. Typhimurium has been shown to occur in response to nitric oxide, an important chemical mediator of the mammalian innate immune response. However, cellular manganese can rapidly return to prechallenge levels, strongly suggesting that one or more S. Typhimurium exporters may contribute to this process. Here, we report that mntP and yiiP contribute to manganese resistance and export in S. Typhimurium. YiiP, also known as FieF, has previously been associated with zinc and iron transport, although its physiological role remains ambiguous due to a lack of zinc-sensitive phenotypes in yiiP mutant strains of S. Typhimurium and E. coli. We report that S. Typhimurium ΔmntP ΔyiiP mutants are exquisitely sensitive to manganese and show that both YiiP and MntP contribute to manganese efflux following nitric oxide exposure.

IMPORTANCE Transition metal cations are required for the function of many proteins but can mediate toxicity when present in excess. Identifying transporters that facilitate metal ion export, the conditions under which they are expressed, and the role they play in bacterial physiology is an evolving area of interest for environmental and pathogenic organisms. Determining the native targets of metal transporters has proved challenging since bioinformatic predictions, in vitro transport data, and mutant phenotypes do not always agree. This work identifies two transporters that mediate manganese efflux from the Gram-negative pathogen Salmonella enterica serovar Typhimurium in response to manganese overload and nitric oxide stress. While homologues of MntP have been characterized previously, this is the first observation of YiiP contributing to manganese export.

KEYWORDS Salmonella, efflux pumps, manganese, metal ion homeostasis, nitric oxide
manganese inhibits heme biosynthesis while chronic manganese stress ultimately leads to iron depletion and impaired formation of Fe-S cluster proteins (15, 16). Together, these effects can result in inhibition of energy-generating and biosynthetic pathways. In *Bacillus subtilis*, which requires manganese for growth, excess manganese has been associated with impaired function of the cytochrome aa₃, heme-copper menaquinol oxidase (QoxABCD) of the electron transport chain (17). Therefore, while increased cellular manganese may benefit bacteria when challenged with a specific physiological or chemical stress, efflux of manganese may be necessary upon alleviation of the aforementioned stress.

Three types of manganese efflux systems have been identified in prokaryotes to date. Transporters from the cation diffusion facilitator (CDF) family are widely prevalent among prokaryotic species with family members implicated in transport of zinc, cadmium, cobalt, nickel and manganese, depending on the sequence motifs present in metal binding regions (18). The prokaryotic CDF manganese exporter MntE was first identified in *Streptococcus pneumoniae* and has since been studied in *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pyogenes* (19–22). *B. subtilis* relies on two CDF family transporters for manganese efflux with MneP functioning as the primary exporter and MneS playing a secondary role (23). Pᵢ₈ ATPases have been shown to export a range of transition metal ions, with manganese export first established for CtpC from *Mycobacterium tuberculosis* (24). MntP, which lacks homology to other established classes of manganese exporters, was first characterized in *Xanthomonas oryzae*, *E. coli*, and *Neisseria meningitidis* (as MntX) (25–27). This architecturally distinct transporter has since been shown to have orthologs in additional species.

Previously, we showed that manganese acquisition by MntH, SitABCD, and ZupT is important for *S. Typhimurium* nitrosative stress resistance. Furthermore, total cellular manganese was restored to prechallenge levels following the resolution of the stress (14). In this study, we sought to identify and characterize *S. Typhimurium* efflux systems that contribute to manganese homeostasis in response to stress. We show that orthologs of the *E. coli* transporters MntP and YiiP protect *S. Typhimurium* against manganese intoxication and mediate manganese efflux during the late-stage response to nitrosative stress.

**RESULTS**

**STM1834 (MntP) protects *S. Typhimurium* against excess manganese.** *E. coli* and *S. Typhimurium* share similar genetic sequences at many loci, so the *S. Typhimurium* genome was searched for proteins with homology to MntP from *E. coli*. One match, at locus *stm1834* (Fig. 1A), was identified with 91% identity and 96% similarity (over 188 amino acids) to *E. coli* MntP. To determine the function of the putative *S. Typhimurium* mntP ortholog, a deletion mutant was generated. The mutant was then grown in the presence of 0.5 mM MnSO₄ and the phenotype compared to the wild-type parental strain. The *S. Typhimurium* ΔmntP strain was delayed for growth in excess manganese compared to the wild-type (Fig. 1B). Constitutive expression of *S. Typhimurium* mntP from a low-copy-number plasmid (pMntP) complemented the growth defect of a ΔmntP mutant (Fig. 1C). Consistent with the growth phenotype data, spot plate assays on manganese supplemented media showed that the *S. Typhimurium* ΔmntP strain had decreased growth on 0.5 mM MnSO₄ and decreased survival on 1 mM MnSO₄ while expression of mntP complemented these phenotypes (Fig. 1D). Taken together, these data are consistent with previous studies of MntP function in *E. coli* and suggest that *stm1834* encodes a manganese exporter homologous to *E. coli* MntP (26).

**MntP is not solely responsible for manganese efflux following NO treatment.** To determine if manganese export by MntP is responsible for returning cellular manganese levels to pretreatment levels following NO exposure, we compared the phenotypes of wild-type and ΔmntP *S. Typhimurium* by inductively coupled plasma-mass spectrometry (ICP-MS). Here, we used conditions defined in our prior studies of *S. Typhimurium* wherein the application of NO stress alters metal homeostasis and induces manganese accumulation (14, 28). Accordingly, cultures were treated with 2 mM diethylamine NONOate (DEANO), a fast-release NO donor, and the cellular manganese content was monitored over the course of 60 min. Consistent with prior observations, manganese levels increased by 30 min posttreatment and
then returned to pretreatment levels by 60 min (Fig. 2) (14). Notably, manganese levels in the DmntP strain were not significantly different than in the wild-type at any time. These data indicate either that manganese efflux does not occur via MntP following NO/C1 treatment or that, in the absence of mntP, manganese efflux occurs via another transporter in S. Typhimurium.

ImiP expression enhances zinc toxicity in S. Typhimurium. Since the S. Typhimurium genome contained only one MntP homologue, the genome was next searched for proteins with homology to the CDF family manganese transporter MntE. Two S. Typhimurium
proteins were observed to have similarity to S. pneumoniae MntE. YiiP (STM4061) had the greatest similarity with 28% identity and 50% similarity over 264 amino acids. In addition to homology with MntE, YiiP also shares homology (26% identity and 51% similarity over 183 amino acids) with CzcD, a well-studied CDF family zinc transporter (Fig. 3A). ZitB (STM0758), a zinc transporter in E. coli and S. Typhimurium, had 20% identity and 48% similarity over 201 amino acids compared to S. pneumoniae MntE (28, 29).

E. coli YiiP, also known as FieF, was first reported to serve as an iron efflux transporter. Subsequent in vitro studies showed that YiiP also had the ability to interact with zinc ions, although its physiological contribution to zinc homeostasis remains to be defined (30–33). Three metal binding regions, the A-site, B-site, and C-site (comprised of C1 and C2 plus a linker) have been identified based on the E. coli YiiP crystal structure (31, 32, 34). The A-site has been established as the primary motif determining metal selectivity (34, 35). S. pneumoniae MntE has an ND-DD A-site motif, but DD-DD A-site motifs are also common in manganese exporting CDF proteins (36). By contrast, the zinc exporting CDF from S. pneumoniae, CzcD, has an HD-HD motif, which is the most common motif in CDF family zinc transporters (36). S. Typhimurium YiiP has a DD-HD A-site motif, which precludes bioinformatic prediction of the native ligand but is suggestive of the potential to export ions other than zinc.

Zinc sensitivity phenotypes have not been observed for S. Typhimurium or E. coli strains with yiiP deleted alone or in combination with other known zinc exporters (28, 29, 37). However, these studies could have failed to detect zinc sensitivity phenotypes if yiiP was not expressed under the conditions tested. To address this, we expressed yiiP constitutively from a low-copy-number plasmid (pYiiP) in the S. Typhimurium DmntA DzitB genetic background to ascertain whether this could decrease the zinc sensitivity of this mutant strain. We observed that the DmntA DzitB strain was delayed for growth in the presence of 0.125 mM ZnSO4 compared to the wild-type. Expression of yiiP in the DmntA DzitB genetic background abrogated bacterial growth (Fig. 3B). Notably, expression of mntP elicited a similar phenotype in the DmntA DzitB background (Fig. 3C). Spot assays revealed that expression of either YiiP or MntP in the DmntA DzitB genetic background led to decreased survival in the presence of 0.0625 mM ZnSO4 and little to no survival on plates with 0.125 mM ZnSO4 (Fig. 3D). These data indicate that YiiP does not facilitate zinc export. It therefore follows that the increased sensitivity of the yiiP-expressing DmntA DzitB strain suggests that YiiP may export a different metal ion that results in enhanced susceptibility to zinc intoxication.

Metal availability results in altered expression of mntP but not yiiP. We next measured yiiP expression under conditions of metal limitation, metal stress, and NO- challenge compared to mntP. In the presence of general divalent cation chelator ethylenediaminetetraacetic acid (EDTA), expression of neither mntP nor yiiP was significantly altered (Fig. 4A). In response to metal stress, mntP was upregulated when the medium was supplemented with...
0.5 mM iron or manganese, but not zinc (Fig. 4B). By contrast, yiiP expression did not change in response to iron, manganese, or zinc (Fig. 4B), which also differs from observations of the orthologous gene from *E. coli* (29, 30). Expression of neither mntP nor yiiP was significantly altered at any time following treatment with 2 mM DEANO (Fig. 4C).

**FIG 3** Expression of YiiP, a protein with homology to MntE and CzcD, enhances zinc sensitivity of an *S. Typhimurium* zinc efflux mutant strain. (A) Alignment of amino acid sequences for *S. Typhimurium* YiiP (STM_YiiP) and the related CDF proteins *S. pneumoniae* MntE (SP_MntE, SP_1552) and *S. pneumoniae* CzcD (SP_CzcD, SP_1857). Identical residues are shown in black, while similar residues are shown in gray. Metal-binding sites from the YiiP crystal structure (PDB ID: 2QFI) are shown. Ligands shared by all proteins are in green boxes. Red boxes denote ligands shared between YiiP and MntE. Blue boxes denote ligands shared between YiiP and CzcD. Boxes are staggered where the amino acid sequence alignment did not match previously assigned locations for two CzcD C-site residues. (B) Growth of *S. Typhimurium* wild-type (WT) pJK724, ΔzntA ΔzitB pJK724, and ΔzntA ΔzitB pYiiP strains in LB with or without 0.125 mM ZnSO4 supplementation. The ΔzntA ΔzitB pJK724 strain was delayed exiting lag phase compared to WT pJK724 in 0.125 mM ZnSO4 (*P* < 0.001), while the ΔzntA ΔzitB pYiiP strain failed to grow. (C) Growth of *S. Typhimurium* wild-type (WT) pJK724, ΔzntA ΔzitB pJK724, and ΔzntA ΔzitB pMntP strains in LB with or without 0.125 mM ZnSO4 supplementation. Data for the growth curves (B) and (C) are the mean of 3 independent experiments. Statistical significance of the difference between the mutant strains and the wild-type was determined using the time (hr) to reach 50% maximum growth (OD600; dashed line) and an unpaired two-tailed *t* test. (D) Growth of strains from (B) and (C) were assessed by spot assays. Dilutions of OD600 = 0.3 cultures were spotted onto LB agar supplemented with ZnSO4 as shown. Zinc efflux mutants expressing YiiP or MntP show decreased survival in the presence of ZnSO4. A representative spot assay for each condition is shown, selected from 3 independent replicates.

0.5 mM iron or manganese, but not zinc (Fig. 4B). By contrast, yiiP expression did not change in response to iron, manganese, or zinc (Fig. 4B), which also differs from observations of the orthologous gene from *E. coli* (29, 30). Expression of neither mntP nor yiiP was significantly altered at any time following treatment with 2 mM DEANO (Fig. 4C).
YiiP contributes to manganese resistance in *S. Typhimurium*. Although expression of yiiP did not change in response to metal stress under our experimental conditions, the protein may be present due to constitutive expression and contribute to metal ion efflux. Building on the observations that YiiP shares homology with MntE (Fig. 3A) and its expression enhanced the zinc sensitivity of a zinc efflux mutant strain in a similar fashion to expression of MntP (Fig. 3B to D), a role in manganese efflux was investigated. To assess whether YiiP contributes to manganese resistance of *S. Typhimurium*, ∆yiiP and ∆mntP ∆yiiP strains were generated. The ∆yiiP strain grew similarly to the wild-type in the presence of 0.5 mM MnSO₄ (Fig. 5A). However, supplementation of the growth medium with 0.5 mM MnSO₄ delayed the growth of the ∆mntP strain and abrogated growth of the ∆mntP ∆yiiP strain (Fig. 5A). In spot assays, the ∆mntP ∆yiiP strain displayed decreased growth at 0.25 mM MnSO₄ and decreased survival at both 0.5 mM and 1 mM MnSO₄. By contrast, the ∆mntP strain only showed a moderate decrease in survival at 1 mM MnSO₄ (Fig. 5C). Taken together, these data show an enhancement of manganese sensitivity when both transporters are absent. Plasmid-based complementation with either mntP or yiiP expressed from the native promoter attenuated the growth defect of a ∆mntP ∆yiiP mutant, although the ∆ntP ∆yiiP plasmid strain still had a minor growth delay relative to the wild-type and ∆mntP ∆yiiP plasmid strains (Fig. 5B). Similar complementation results were obtained in spot assays (Fig. 5D).

*5. Typhimurium* mutants lacking *mntP* and *yiiP* are neither sensitive to zinc nor disrupted for zinc homeostasis. Previous experiments investigating the metal binding properties of recombinant *E. coli* YiiP revealed a capacity to interact with zinc ions *in vitro*, but not manganese (33, 38). Thus, the observed impact of manganese on the growth and survival of the *S. Typhimurium* ∆mntP ∆yiiP strain may reflect an indirect effect on zinc homeostasis. Interplay between zinc and manganese homeostasis has been shown to occur in several Gram-positive pathogens, such as *S. pneumoniae* where zinc has been established to disrupt manganese uptake and increase sensitivity to oxidative stress (39–42). However, this phenomenon has not been reported for the *Enterobacteriaceae* and, in *S. Typhimurium*, this may be attributable to the presence of the manganese-transporting natural resistance-associated macrophage protein (NRAMP) transporter MntH, which is not susceptible to zinc inhibition (9). By contrast, the impact of manganese on zinc homeostasis in *S. Typhimurium* has not been determined. Accordingly, we investigated whether the observed manganese sensitivity of the *S. Typhimurium* ∆mntP ∆yiiP strain was due to pleiotropic effects of perturbed zinc homeostasis.

Here, we examined the impact of zinc stress on the wild-type ∆yiiP, ∆mntP, and ∆mntP ∆yiiP strains. This revealed that zinc supplementation had no impact on the growth phenotype of any strain (Fig. 6A and B). These data indicate that *S. Typhimurium* zinc homeostasis is not dysregulated in the absence of manganese efflux. Furthermore, neither YiiP nor MntP contributes to substantially to zinc homeostasis. To further probe the impact on the zinc regulatory network of *S. Typhimurium*, the expression of genes controlled by the zinc uptake
regulator, Zur, and zinc export regulator, ZntR, were analyzed in the wild-type and ΔmntP ΔyiiP strains during exposure to excess manganese. The sensitivity of these metalloregulators is in the femtomolar to nanomolar range; thus, they provide a highly sensitive insight into cellular zinc homeostasis (43–45). Here, we monitored the expression of the Zur-regulated zinc uptake transporter znuABC, the primary S. Typhimurium pathway for zinc acquisition (46–48); the ZntR-regulated zinc exporter zntA, the major S. Typhimurium zinc efflux system (28, 49, 50); and zupT, which has been implicated in zinc and manganese import, although its regulatory control remains to be defined (14, 51, 52). These data show that there was no significant difference in expression of zntA, znuC, or zupT between the wild-type and ΔmntP ΔyiiP strains at 20 min (Fig. 6C) or 60 min (Fig. 6D) in the presence of

FIG 5 Manganese sensitivity of S. Typhimurium is enhanced in the absence of both mntP and yiiP. (A) Growth phenotypes of S. Typhimurium wild-type (WT), ΔmntP, ΔyiiP, and ΔmntP ΔyiiP strains in LB with or without 0.5 mM MnSO₄ supplementation. The ΔmntP strain was delayed exiting lag phase compared to WT in LB supplemented with 0.5 mM MnSO₄ (P < 0.001), and the ΔmntP ΔyiiP strain failed to grow. (B) Growth phenotypes of S. Typhimurium empty vector strains WT pRB3 and ΔmntP ΔyiiP pRB3 compared to plasmid-based complementation strains ΔmntP ΔyiiP pMntP and ΔmntP ΔyiiP pYiiP in LB with or without 0.5 mM MnSO₄ supplementation. The expression constructs pMntP and pYiiP use the native promoters. Growth of ΔmntP ΔyiiP pYiiP was delayed relative to WT (P < 0.001). Data for the growth curves (A) and (B) are the mean of 3 independent experiments. Statistical significance of the difference between the mutant strains and the wild-type was determined using the time (hr) to reach 50% maximum growth (OD₆₀₀; dashed line) and an unpaired two-tailed t test. (C) Growth of strains from (A) by spot plate assays. Dilutions of OD₆₀₀ = 0.3 cultures were spotted onto LB agar supplemented with MnSO₄ as shown. The ΔmntP ΔyiiP strain displayed decreased growth at 0.25 mM MnSO₄ and decreased survival at 0.5 mM and 1 mM. (D) Growth of strains from (B) by spot plate assays. Dilutions of OD₆₀₀ = 0.3 cultures were spotted onto LB agar supplemented with MnSO₄ as shown. A representative spot assay for each condition is shown, selected from 3 independent replicates. Expression of either YiiP or MntP complemented the survival defect of the ΔmntP ΔyiiP strain in the presence of MnSO₄.

regulator, Zur, and zinc export regulator, ZntR, were analyzed in the wild-type and ΔmntP ΔyiiP strains during exposure to excess manganese. The sensitivity of these metalloregulators is in the femtomolar to nanomolar range; thus, they provide a highly sensitive insight into cellular zinc homeostasis (43–45). Here, we monitored the expression of the Zur-regulated zinc uptake transporter znuABC, the primary S. Typhimurium pathway for zinc acquisition (46–48); the ZntR-regulated zinc exporter zntA, the major S. Typhimurium zinc efflux system (28, 49, 50); and zupT, which has been implicated in zinc and manganese import, although its regulatory control remains to be defined (14, 51, 52). These data show that there was no significant difference in expression of zntA, znuC, or zupT between the wild-type and ΔmntP ΔyiiP strains at 20 min (Fig. 6C) or 60 min (Fig. 6D) in the presence of
manganese. Thus, the S. Typhimurium DmntP DyiiP manganese-induced growth and survival defects (Fig. 5) are not an indirect effect arising from disrupted zinc homeostasis.

We next investigated whether YiiP participates in control of cellular manganese levels following exposure to NO. It is important to note that in this experiment, direct comparisons are confined to the wild-type and the mutant strains within each analysis. This is due to differences in medium manganese concentrations that differed between the analyses (i.e., Fig. 7A vs. Fig. 7B), which influenced the absolute cellular abundances in the bacterial strains (43).

Changes in cellular manganese followed generally similar patterns in the wild-type and DyiiP strains following treatment with 2 mM DEANO. However, manganese levels were elevated in the DyiiP strain relative to the wild-type strain at 5, 15, and 60 min (Fig. 7A). Manganese levels were greater in the DmntP DyiiP strain than in the wild-type at 5, 15, 45, and 60 min after treatment with 2 mM DEANO, but both strains reached a similar peak at 30 min (Fig. 7B). In the wild-type strain, cellular manganese decreased at 45 min compared to the 30 min peak and fell to pretreatment levels by 60 min. In the DmntP DyiiP strain, there was no significant difference in manganese levels at 30, 45, and 60 min posttreatment, suggesting that efflux was impaired when both genes were deleted (Fig. 7B).

Residue Asp45 is required for YiiP to alleviate manganese toxicity. Prior work has indicated that the A-site motif of CDF transporters dictates metal specificity (34, 35). For YiiP, the aspartate residue at position 45 has been implicated in facilitating transport of both cadmium and zinc in in vitro assays (35). When the aspartate residue was mutated to histidine (D45H), creating an HD-HD A-site motif in YiiP, the mutant protein transported zinc at similar rates as wild-type YiiP in in vitro assays, but no longer transported cadmium (35). In this study, our data indicate that S. Typhimurium YiiP is associated with physiological manganese export and do not support a role in zinc homeostasis.

FIG 6 Zinc sensitivity is not enhanced in a DmntP DyiiP mutant, and expression of zinc-regulated transporters is not perturbed by manganese. (A) Growth of S. Typhimurium wild-type (WT) and the mutant derivatives ΔyiiP, ΔmntP, and ΔmntP ΔyiiP in LB with or without 0.5 mM ZnSO4 supplementation. Data are the mean of 3 independent experiments, and statistical significance was determined by the time (hr) to reach 50% maximum growth (OD600; dashed line) by unpaired two-tailed t test. (B) Growth of strains from (A) assessed using spot assays on LB agar supplemented with ZnSO4 as shown. A representative spot assay for each condition is shown, selected from 3 independent replicates. (C) qPCR analyses of the zinc-regulated genes zntA, znuC, and zupT determined in response to challenge by 0.5 mM MnSO4 (20 min exposure; OD600 = 0.5 cultures). (D) qPCR analyses of the zinc-regulated genes zntA, znuC, and zupT determined in response to challenge by 0.5 mM MnSO4 (60 min exposure; OD600 = 0.5 cultures). Data in (C) and (D) are the mean (± standard deviation) of 3 independent experiments. Statistical significance was determined by a one-sample t test to a hypothetical mean of either 1 or −1. No significant differences were observed.
homeostasis; however, this does not preclude a capacity for interaction with zinc in vitro. Here, we sought to determine the contribution of Asp45 to YiiP manganese transport. We generated a point mutation, substituting histidine for aspartate (D45H) in the p_YiiP plasmid (p_YiiP D45H). The mutant derivative was then investigated in a ΔmntP ΔyiiP p_YiiP D45H strain was no different than the ΔmntP ΔyiiP pYiIP strain was no different

DISCUSSION

Manganese efflux by MntP and the CDF transporter MntE has been studied in a variety of prokaryotic species, but thus far the presence of multiple manganese efflux systems within a single prokaryote has only been characterized in B. subtilis. S. Typhimurium encodes both an MntP homologue and YiiP, a CDF transporter. Identifying the target metal of CDF family transporters can be challenging despite efforts to establish the motifs that provide ion selectivity. YiiP has homology within the metal coordinating A-site to both zinc and manganese-transporting members of the CDF family (Fig. 3A) (36). YiiP from E. coli has been characterized as a zinc transporter using in vitro and structural methods, while an in vivo study linked its function to iron homeostasis. A physiological role for YiiP in zinc homeostasis has not yet been established in S. Typhimurium or E. coli, with ΔyiiP mutant strains not demonstrating zinc susceptible phenotypes (28, 29, 37). Here we show that expression of YiiP enhances the zinc sensitivity of ΔzntA ΔzitB S. Typhimurium rather than reducing it (Fig. 3B). These data, along with those showing enhanced manganese sensitivity and lack of manganese export in ΔmntP ΔyiiP S. Typhimurium (Fig. 5, 7B), suggest that zinc is not the cognate cargo of S. Typhimurium YiiP in vivo. Manganese transport phenotypes have been demonstrated in vivo for YiiP homologues

FIG 7 Intracellular manganese levels increase sooner in ΔyiiP and ΔmntP ΔyiiP mutants following NO treatment and do not decrease in ΔmntP ΔyiiP. Total cellular manganese accumulation in wild-type (WT) and mutant S. Typhimurium cultures treated with 2 mM DEANO, determined by ICP-MS. (A) Intracellular manganese was greater in ΔyiiP S. Typhimurium than in WT (*) at 5 (P = 0.004), 15 (P = 0.03) and 60 min (P = 0.001) posttreatment. Manganese was elevated (%) in both WT (P = 0.02) and ΔyiiP (P = 0.009) at 30 min posttreatment compared to 0 min. (B) Intracellular manganese was greater in ΔmntP ΔyiiP S. Typhimurium than in WT (*) at 5 (P = 0.01), 15 (P = 0.04), 45 (P = 0.008), and 60 min (P < 0.001) posttreatment. Manganese was significantly elevated (%) in WT (P < 0.001) and ΔmntP ΔyiiP (P = 0.003) at 30 min posttreatment compared to 0 min. In WT, intracellular manganese was lower at 45 min than at 30 min (P = 0.003) and was no different than at 0 min by 60 min posttreatment. Intracellular manganese was no different in ΔmntP ΔyiiP at 45 and 60 min posttreatment than at 30 min. ICP-MS data are the mean of 4 independent experiments. Error bars represent standard deviation. Statistically significant differences across time points for each strain and between WT and mutant were determined by unpaired two-tailed t test.
from *Sinorhizobium meliloti, Deinococcus radiodurans, and Rhizobium etli* (53–55). These proteins share a clade with YiiP from *E. coli* that is phylogenetically distinct from the clades containing the well-characterized CDF zinc transporters CzcD and ZitB (54, 56). The variety of proposed substrates for transporters within this clade is diverse while relatively few transporters have been experimentally characterized. Further work will be required to gain an accurate understanding of the biological functions of this group of transporters.

While function of MntP alone was sufficient to provide protection from manganese toxicity under the conditions of this study, deletion of both *mntP* and *yiiP* enhanced the sensitivity of *S. Typhimurium* to manganese intoxication (Fig. 5A and C). Function of either MntP or YiiP could restore manganese homeostasis following NO- treatment, and only the ∆*mntP* ∆*yiiP* strain was abrogated for manganese efflux during the late-stage response to NO- exposure (Fig. 7B). Selectivity for manganese depends on residue Asp45 within the A-site metal binding region of YiiP. A change in transport activity in response to mutation of Asp45 is consistent with similar results arising from the mutation of A-sites in *E. coli* YiiP and other CDF transporters (34, 35). While we cannot exclude the possibility that YiiP might facilitate the transport of other transition metal cations under certain conditions, the results of this study support a role for YiiP as a manganese exporter in *S. Typhimurium.*
Regulation of yiiP expression remains an open question. Unlike mntP, it is not part of the MntR regulon and has not been shown to be part of the Fur, Zur, or ZntR regulons (26, 57). Expression of mntP is regulated by both MntR and the ybbP-ykoY manganese-sensing riboswitch in E. coli (58). The promoter region of mntP from S. Typhimurium shows homology to that of E. coli, suggesting that similar regulatory mechanisms may apply to mntP expression in both organisms. By contrast, the promoter region of yiiP lacks this homology. Induction of mntP expression occurred in response to manganese and iron stress, whereas yiiP expression was unaffected by the conditions tested (Fig. 4B). Previous work using β-galactosidase fusions to the yiiP promoter in E. coli showed upregulation in response to zinc and a modest response to iron after several hours of incubation (29, 30). Since none of the known metal-responsive regulators have been implicated in yiiP expression, any expression change in response to metal excess may be indirect and more time may be necessary for the cellular conditions driving regulation to develop. Alternatively, expression of yiiP may be constitutive in S. Typhimurium under standard laboratory growth conditions. Constitutive expression would be consistent with the more rapid accumulation of manganese observed in strains lacking yiiP (Fig. 7), while a strain lacking only the inducible mntP acquires manganese similarly to the wild-type (Fig. 2). Expression of mntE, the CDF manganese transporter from S. pneumoniae, does not respond to manganese abundance and has been proposed to be expressed constitutively (19). Manganese efflux activity in E. coli is further regulated by the small protein MntS. E. coli that overexpress MntS display enhanced manganese accumulation and have the same manganese-sensitive phenotype as ΔmntP mutants, suggesting that this small protein may function to prevent the efflux activity of MntP (16). Overexpression of MntS in a ΔmntP strain did not enhance the manganese sensitivity of this mutant, suggesting that it does not inhibit the function of additional manganese exporters such as YiiP, but this possibility has not yet been investigated directly.

While efflux by either MntP or YiiP is sufficient to restore baseline manganese levels in S. Typhimurium following NO exposure, and both contribute to protecting S. Typhimurium from excess manganese in culture, the broader functional significance of these transporters in S. Typhimurium biology and pathogenesis has yet to be determined. Manganese acquisition is required for S. Typhimurium infection and virulence, but what role, if any, efflux might play is not currently known (10, 11). Manganese efflux has been shown to play an important role in the virulence of other pathogens such as S. pneumoniae and S. aureus and in colonization by E. faecalis (19–21). Although manganese has primarily been understood to act as an antioxidant, and absence of mntE rendered S. pneumoniae more resistant to both NO- and oxidative stress in the form of methyl viologen, this has not been the case for all organisms and oxidants (19). S. aureus lacking mntE was more sensitive to sodium hypochlorite (NaOCl), while S. pyogenes was more sensitive to peroxide (H2O2) (20, 22). Manganese excess has been shown to affect electron transport chain function and function of Fe-S cluster enzymes in central energy-generating pathways. (15–17). These proteins are also targets of oxidants, which could explain, at least in part, why enhanced sensitivity to oxidants is seen for some species, but the mechanisms underlying the requirement for manganese efflux remain incompletely understood. In light of these data, the dynamic flux of multiple metals that occurs in response to NO-, and the varied environments and stresses encountered by pathogens within the host, characterizing the biological roles of microbial manganese efflux systems, is an open and potentially complex area of investigation. Based on the findings of this study, we suggest that any future work concerning the biological significance of manganese efflux in S. Typhimurium should address both the function of MntP and the newly established manganese export function of YiiP.

MATERIALS AND METHODS

Bioinformatic analysis. S. Typhimurium homology matches to MntP from Escherichia coli MG1655 and MntE and CsdD from Streptococcus pneumoniae TIGR4 were determined using BLASTP from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website with default settings. Alignments were generated using the KEGG ClustalW tool (59). Alignment graphics were generated using BoxShade hosted by ExPASy, the Swiss Institute of Bioinformatics Resource Portal (https://embnet.vital-it.ch/software/BOX_form.html).

Growth conditions. Salmonella enterica serovar Typhimurium and Escherichia coli were grown in Luria-Bertani medium (LB; Fisher) at 37°C with shaking at 250 rpm unless otherwise specified. Antibiotic selection
Table 1: Strains and plasmids

| Plasmid or strain | Genotype | Source |
|-------------------|----------|--------|
| pKD46             | bla araC-P<sub>mntP</sub> exo oriR101 repA101ts | (60) |
| pKD3              | bla FRTcatFRT PS1 PS2 oriRy | (60) |
| pKD4              | bla FRTaphFRT PS1 PS2 oriRy | (60) |
| pCP20             | bla cat ci857 IPr flp PSC101 oriTS | (60) |
| pRB3-273C         | bla par RK2 oriV trfA | (62) |
| (pRB3)            | bla par RK2 oriV trfA | (63) |
| pJK724            | bla par RK2 oriV trfA P<sub>mntP</sub> yiiP | This study |
| pEF101            | bla par RK2 oriV trfA P<sub>mntP</sub>yiiP | This study |
| (p_MntP)          | bla par RK2 oriV trfA P<sub>mntP</sub>yiiP | This study |
| pEF102            | bla par RK2 oriV trfA P<sub>mntP</sub>yiiP | This study |
| (p_YiiP)          | bla par RK2 oriV trfA P<sub>mntP</sub>yiiP | This study |
| pEF106            | bla par RK2 oriV trfA P<sub>mntP</sub>yiiP | This study |
| (pMntP)           | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP | This study |
| pEF108            | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP D45H | This study |
| (p_YiiP D45H)     | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP D45H | This study |
| pEF115            | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP:FLAG | This study |
| (p_YiiP:FLAG)     | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP D45H:FLAG | This study |
| pEF116            | bla par RK2 oriV trfA P<sub>YiiP</sub>yiiP D45H:FLAG | This study |

Strain and plasmid construction. All strains and plasmids are listed in Table 1. All primers are listed in Table 2. S. Typhimurium strains were generated in the ATCC 14028s genetic background, which also served as the wild-type strain in this study (JK237). Deletion mutants were constructed using λ-RED mediated recombination with either pKD3 or pKD4 as the template (60). Expression of Flp-FRT recombinase from pCP20 was used to remove the antibiotic resistance cassettes from strains EF657 and EF635 to generate strains EF725 and EF755 (60). Combination mutants were created using P22HT105/int bacteriophage transduction (61). All mutants were verified by PCR.

E. coli strain TB1 was used as the cloning host strain. Purified genomic DNA from S. Typhimurium 14028s was used as the PCR template unless otherwise specified. Plasmids pEF101 and pEF102 were generated by amplifying the upstream promoter region and coding sequence of mntP using primers EFP334 and EFP335 and yiiP using primers EFP336 and EFP340. Amplified fragments were digested with...
KpnI and HindIII then ligated into pRB3-273C digested with the same enzymes in reverse orientation to the multiple cloning site promoter (62). Constructs were confirmed by sequencing with JKP227 and JKP244 then transformed into strain EF657 to generate strains EF744 and EF745. To generate plasmids pEF103 and pEF106, the coding sequence of yiiP was amplified using primers EFP319 and EFP320 and the coding sequence of mntP was amplified using primers EFP346 and EFP335. Amplified fragments were digested with NcoI and HindIII then ligated into pJK724 digested with the same enzymes (63). Constructs were sequenced using primers EFP21 and JKP777 then transformed into zinc efflux mutant strain EF528 to generate strains EF746 and EF762. To generate plasmid pEF108, primers EFP336 and EFP347 were used to amplify a short fragment containing the target mutation (D45H). The fragment was purified from an agarose gel then used in a second amplification reaction with EFP340. Full-length product was digested with KpnI and HindIII then ligated into pRB3-273C digested with the same enzymes in reverse orientation to the multiple cloning site promoter. The construct was verified by sequencing with JK727 and JK244 then transformed into strain EF657 to generate strains EF773. Plasmids pEF115 and pEF116 were generated by amplifying the coding sequences of plasmids pEF102 and pEF108 using primers EFP319 and EFP397. Amplified fragments were digested with NcoI and HindIII then ligated into pJK724 digested with the same enzymes. Constructs were sequenced using primers EFP21 and JKP777 then transformed into strain EF657 to generate strains EF829 and EF830.

**Metal sensitivity growth curve assays.** To determine manganese sensitivity, wild-type S. Typhimurium (JK237) and transporter mutants (EF561, EF635, EF657) were grown overnight in LB, normalized to optical density at 600 nm (OD600) of 1, and diluted in triplicate 1:1000 into LB or LB with 0.5 mM MnSO4 for a final volume of 300 μL in a flat-bottom 96-well nontreated tissue culture microtiter plate (Midwest Scientific). Cultures were grown aerobically with shaking at 367 rpm (3 mm) in a Biotek Synergy HTX multimode 96-well plate reader at 37°C. Growth was monitored by recording OD600 every 15 min for 40 h. Statistical significance was determined by comparing the time required to reach 50% maximum OD600 by unpaired two-tailed t test using Microsoft Excel.

**TABLE 2 Primer sequences**

| Primer  | Sequence 5′–3′                                | Purpose                   |
|---------|-----------------------------------------------|---------------------------|
| EFP21   | CGACATCATAAACGGTTTCTGCC                     | Sequencing of pEF103, pEF106 |
| EFP300  | ATGTTCGAGGGGCGATGATTGTTAACCTGGATACCC        | Creation of plasmid EF635 |
| EFP301  | CGGTGTAACCAGTGAGTTGCTTTC                      | Creation of plasmid EF635 |
| EFP306  | ATGTTAATTTTGCGCCGTCATTG                      | Creation of plasmid EF635 |
| EFP319  | CACCATGCGCATTGAACATACACTGGAGCTGACC          | Creation of plasmid EF635 |
| EFP320  | CACAAAGTTTATACAAAGCTGAACCTCAGCCCCC          | Creation of plasmid EF635 |
| EFP334  | GCGGTACCAACCTCATTAGAATACACATATC             | Creation of plasmid EF635 |
| EFP335  | ATAAAGTTTTAACCGGAAATGCGTACC                 | Creation of plasmid EF635 |
| EFP336  | ATAGGTACCCCCCGTGGTTCCTGCTGACAGCC            | Creation of plasmid EF635 |
| EFP340  | GCGAAGTTTATACAAAGCTGAACCTCAGCCCCC          | Creation of plasmid EF635 |
| EFP346  | AACCATGGAATGTTGCTGGGCGAGT                 | Creation of plasmid EF635 |
| EFP347  | AATATCCACACGCAGTGACCAACC                   | Creation of plasmid EF635 |
| EFP397  | CACCAAGCTTTACTGTCATGCTTCTGTAGCTAGCT        | Creation of plasmid EF115, EF116 |
| JKP227  | ACTTATAGGCAAACCCGAGG                      | Sequencing of pEF101, pEF102 |
| JKP244  | CTCTCTGCTATTACCGACAGG                      | Sequencing of pEF101, pEF102 |
| JKP744  | CCTATGGACGCGCCGTCATTAGCGGCCGGCGATGCTGAGCGTCCCT | Creation of plasmid EF560, EF561 |
| JKP745  | AATGACATCTGAAACCGGAAAACCGCTGAAATACGCTGACAGCC | Creation of plasmid EF560, EF561 |
| JKP746  | TGAACATGACACCAAGGAA                          | Creation of plasmid EF560, EF561 |
| JKP747  | CCGATTTCCTTAATCATGACTACC                    | Creation of plasmid EF560, EF561 |
| JKP777  | CACTTCTGAGCGGTCGAGGG                        | Sequencing of pEF103, pEF106 |
| rpoD qPCR fwd | GTGAAATGGGACCTGTTGAAAC                 | rpoD qPCR |
| rpoD qPCR rev | TCCCAAGCAGTAAGTAAAGG                      | rpoD qPCR |
| yiiP qPCR fwd | ACTGCTGCTAGTTATGAGCC                        | yiiP qPCR |
| yiiP qPCR rev | ACAGAAACAACGCGGACCG                          | yiiP qPCR |
| mntP qPCR fwd | GCTGAGGCTGTCTTACTTTGCC                     | mntP qPCR |
| mntP qPCR rev | GCTATCACTGTTGCTTTCCGCC                     | mntP qPCR |
| zntA qPCR fwd | TCTGTATCCTATTGCCC                           | zntA qPCR |
| zntA qPCR rev | CAATAACACCGTGACCCCCAAGT                    | zntA qPCR |
| znuC qPCR fwd | GGAGAAATCCAGGCTTCTGACC                    | znuC qPCR |
| znuC qPCR rev | TTTCGCGGACGATAGCCGATA                      | znuC qPCR |
| zupT qPCR fwd | GATCATGCTGTTTCTGGCTGCT                     | zupT qPCR |
| zupT qPCR rev | CCAGATCCTTGCGGATAGGCG                      | zupT qPCR |
Complementation of ΔmntP was assessed using strains JK895, EF737, and EF736. Complementation of ΔmntP ΔyiiP was assessed using JK895, EF739, EF740, and EF741. Complementation of ΔmntP ΔyiiP with the YiiP D45H mutant construct pEF108 was assessed using strains JK895, EF739, EF741, and EF773. Growth assays were carried out as described above except MnSO₄ was added at both 0.5 mM and 0.25 mM final concentration.

Strains used to determine zinc sensitivity in response to YiiP and MntP expression were EFS64, EF745, EF746, and EF762. Zinc sensitivity of manganese transporter mutants was assessed using strains JK237, EF635, and EF657. Zinc sensitivity assays were carried out as described above but the LB was supplemented with 0.125 mM or 0.5 mM ZnSO₄ respectively.

**Metal sensitivity spot assays.** Strains were grown overnight in LB, diluted 1:1000 in 5 mL fresh medium then grown with shaking at 37°C for 3 h to OD₆₀₀ = 0.3. Cultures were serially diluted 10-fold in PBS, then 3 µL were spotted onto LB agar plates with or without metal sulfate supplementation. Plates were grown 14–16 h at 37°C prior to imaging. Spot assays utilized the same strains as growth curve assays. Complementation of manganese sensitivity by expression of FLAG-tagged YiiP proteins was assessed using strains EFS64, EF747, EF829, and EF830.

**Metal content analyses.** Inductively coupled plasma-mass spectrometry (ICP-MS) analyses were conducted using strain variants that were also flagellar mutants (EF610, EF697, EF698, EF701) to increase pelleting efficiency as in previous studies (14, 28). Overnight cultures were diluted 1:1000 into 100 mL fresh LB medium and then grown to OD₆₀₀ ~ 1. Cultures were divided into 5 mL aliquots in 18 × 150 mm glass tubes, treated with 2 mM diethylamine NONOate (DEANO) and returned to shaking at 37°C. At 0, 5, 15, 30, 45, and 60 min posttreatment, 4.5 mL of culture was pelleted by centrifugation then washed twice with ultrapure water. Pellets were resuspended in analytical grade nitric acid, boiled, then diluted 1:10 with ultrapure water for analysis on an Agilent 8900x QQQ ICP-MS. Bacterial numbers, determined as CFU, were enumerated at each time point for calculation of relative metal concentrations. Statistical significance was determined by two-tailed t test in Microsoft Excel.

**Gene expression analysis.** Primer sequences used for expression analysis are listed in Table 2. For expression under metal chelation, wild-type S. Typhimurium was grown overnight, diluted 1:1000 in 2 × 5 mL fresh LB medium, and grown to OD₆₀₀ ~ 1. Ethylenediaminetetraacetic acid (EDTA) at a concentration of 3 mM was added to one of the cultures for 20 min. 1.5 mL of each culture was pelleted by centrifugation then resuspended in 800 µL TRIzol for RNA isolation.

For analysis of expression in response to metal supplementation, overnight LB cultures were diluted 1:1000 into 25 mL modified Tris minimal medium (50 mM Tris, 80 mM NaCl, 2 mM KCl, 5 mM NH₄SO₄, 1.65 mM NaSO₄·10H₂O, 1 mM MgSO₄·6H₂O, 0.3 mM CaCl₂, 1.6 mM Na₂HPO₄, 0.2% glucose, 3 g.L⁻¹ casamino acids) and grown to OD₆₀₀ ~ 0.5. The culture was subdivided into 4 × 5 mL cultures in 18 × 150 mm glass tubes. One was left untreated and the others were supplemented with 0.5 mM FeSO₄ plus 1 mM ascorbate, 0.5 mM MnSO₄, or 0.5 mM ZnSO₄. After 20 min, 3 mL of culture was pelleted by centrifugation and pellets were resuspended in 800 µL TRIzol for RNA isolation.

To monitor expression in response to nitrosative stress, wild-type S. Typhimurium was grown overnight, diluted 1:1000 in 100 mL fresh LB medium, and grown to OD₆₀₀ ~ 1. The culture was subdivided into 5 duplicate pairs of 5 mL cultures in 18 × 150 mm glass tubes. One set was treated with 2 mM DEANO and one set left untreated. Cultures were returned to shaking at 37°C. At 5, 15, 30, 45, and 60 min posttreatment, 1.5 mL of each culture was pelleted by centrifugation then resuspended in 800 µL TRIzol for RNA isolation.

To determine whether supplementation of growth medium with manganese disrupts zinc homeostasis and expression of zinc-regulated zinc transporters, JK237 and EF657 S. Typhimurium were grown overnight, diluted 1:1000 in 2 × 5 mL fresh LB medium, and grown to OD₆₀₀ ~ 0.5. One culture was left untreated and the other was supplemented with 0.5 mM MnSO₄ before cultures were returned to shaking at 37°C. At 20 and 60 min posttreatment, 1.5 mL of treated and untreated culture was pelleted by centrifugation then resuspended in 800 µL TRIzol for RNA isolation.

For all analyses, RNA and cDNA were prepared as described previously (64). Quantitative PCR (qPCR) was carried out using SYBR green on a Bio-Rad CFX96 real-time system with rpoD as the internal control for normalization. Fold change values (treated/untreated) were log 2 transformed prior to plotting. Statistical significance was determined by one-sample t test compared to a hypothetical means of 1 or 1 using GraphPad Prism.

**Western blotting.** Strains EF829 and EF830 were grown as for spot assays, then 3 mL of culture was pelleted, resuspended in 100 µL PBS, and diluted 1:1 in 2 × Laemmli sample buffer (Bio-Rad) with DTT (100 mM final). Samples were incubated 60 min at 55°C before 7.5 µL was loaded on a 4–20% Tris-glycine gel for separation in Tris-glycine-SDS buffer (Bio-Rad). Separated proteins were transferred to nitrocellulose membranes, which were blocked with EveryBlot Blocking Buffer (Bio-Rad) then probed with 1:000 monoclonal anti-FLAG M2-peroxidase (HRP) antibody (Millipore Sigma). Blots were visualized using an ECL Western blotting analysis system (Amersham) on an ImageQuant LAS 4000 imaging system (GE Healthcare) that captures both chemiluminescent and visible images. The visible image of the protein size standards was aligned with the chemiluminescent image of the same blot.

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