Functional Determinants of Metal Ion Transport and Selectivity in Paralogous Cation Diffusion Facilitator Transporters CzcD and MntE in *Streptococcus pneumoniae*

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ABSTRACT

Cation diffusion facilitators (CDFs) are a large family of divalent metal transporters that collectively possess broad metal specificity and contribute to intracellular metal homeostasis and virulence in bacterial pathogens. *Streptococcus pneumoniae* expresses two homologous CDF efflux transporters, MntE and CzcD. Cells lacking *mntE* or *czcD* are sensitive to manganese (Mn) or zinc (Zn) toxicity, respectively, and specifically accumulate Mn or Zn, respectively, thus suggesting that MntE selectively transports Mn, while CzcD transports Zn. Here, we probe the origin of this metal specificity using a phenotypic growth analysis of pneumococcal variants. Structural homology to *Escherichia coli* YiiP predicts that both MntE and CzcD are dimeric and each protomer harbors four pairs of conserved metal-binding sites, termed the A site, the B site, and the C1/C2 binuclear site. We find that single amino acid mutations within both the transmembrane domain A site and the B site in both CDFs result in a cellular metal sensitivity similar to that of the corresponding null mutants. However, multiple mutations in the predicted cytoplasmic C1/C2 cluster of MntE have no impact on cellular Mn resistance, in contrast to the analogous substitutions in CzcD, which do have an impact on cellular Zn resistance. Deletion of the MntE-specific C-terminal tail, present only in Mn-specific bacterial CDFs, resulted in only a modest growth phenotype. Further analysis of MntE-CzcD functional chimeric transporters showed that Asn and Asp in the ND-DD A-site motif of MntE and the most N-terminal His in the HD-HD site A of CzcD (the specified amino acids are underlined) play key roles in transporter metal selectivity.

IMPORTANCE

Cation diffusion facilitator (CDF) proteins are divalent metal ion transporters that are conserved in organisms ranging from bacteria to humans and that play important roles in cellular physiology, from metal homeostasis and resistance to type I diabetes in vertebrates. The respiratory pathogen *Streptococcus pneumoniae* expresses two metal CDF transporters, CzcD and MntE. How CDFs achieve metal selectivity is unclear. We show here that CzcD and MntE are true paralogs, as CzcD transports zinc, while MntE selectively transports manganese. Through the use of an extensive collection of pneumococcal variants, we show that a primary determinant for metal selectivity is the A site within the transmembrane domain. This extends our understanding of how CDFs discriminate among transition metals.

Transition metals from manganese (Mn) to zinc (Zn) in the first row of the periodic table serve as essential cofactors in metalloenzymes that are required for many important cellular processes, including replication, regulation, and central metabolism, among all domains of life (1–3). Despite their importance, excess transition metals can impair bacterial growth. Excess iron (Fe) is harmful due to its participation in Fenton chemistry, which produces a highly reactive hydroxyl radical that can damage biomolecules (4–7). Other transition metals, including Mn, Zn, cobalt (Co), nickel (Ni), and copper (Cu), become toxic at high concentrations partly because they compete for each other's cognate metal-binding sites in enzymes (8–17). Regardless of the mechanism of metal toxicity, bacteria have evolved ways to minimize the deleterious impact of metal ion excess. One well-characterized strategy involves the use of metal efflux transporters, such as cation diffusion facilitator (CDFs) (18) and P-type ATPase proteins (19), to remove excess metal ions from the cytoplasm.

CDFs are a large family of divalent metal/H⁺ antiporters that collectively possess broad substrate (metal) specificity and play important roles in global intracellular metal homeostasis in bacteria and in the pathogenicity of bacteria (18, 20–22). CDF transporters are dimeric, with each protomer containing an N-terminal

six-helical transmembrane domain (TMD; transmembrane [TM] helices TM1 to TM6) and a C-terminal mixed α/β cytoplasmic domain (CTD) that varies in length (Fig. 1; see also Fig. S1 in the supplemental material) (18). The most extensively structurally characterized CDF to date is *Escherichia coli* YiiP (FieF), previously shown to protect cells from Zn and Fe toxicity (23, 24). Crystallographic analysis of Zn-bound *E. coli* YiiP in detergent reveals that each protomer harbors four metal-binding sites, designated A, B, C1, and C2 (Fig. 1 and 2) (24, 25). Site A is positioned

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FIG 1 Ribbon representation of the outward-facing structure of *Escherichia coli* YiiP (25) (left) highlighting the metal-binding A, B/charge-interlock, and C1/C2 sites within each protomer (right). Residue numbers correspond to those of *E. coli* YiiP, with the amino acid substitutions of each candidate metal ligand in *S. pneumoniae* CzcD and MntE predicted from a multiple-sequence alignment (Fig. 2; see also Fig. S1 in the supplemental material) being highlighted using the one-letter residue code (Table 1). Color coding is as follows: blue, TMD; magenta, cytoplasmic; red, predicted linker region; yellow, Zn ions.

within the TMD with two ligands derived from each of the TM2 (D45, D49) and TM5 (H153, D157) helices to create a DD-HD A-site motif (Fig. 1; Table 1). Site B ligands are clustered within a short cytoplasm-facing loop between transmembrane helices TM2 and TM3, just N terminal to one of the residues of the inter-subunit charge interlock. The second charge-interlock residue is found just C terminal to TM6 (Fig. 1). The remaining two metal-binding sites, designated C1/C2, comprise a subunit-bridging two-metal binuclear cluster found entirely within the CTD, with the most C-terminal ligand functioning as a bridging carboxylate to the C1 and C2 metals (Fig. 1 and 2).

In vitro biochemical studies have shown that ligands to the transmembrane A site in *E. coli* YiiP are required for metal transport. Further, replacement of the most N-terminal A-site ligand, D45, of the DD-HD motif (D45 is in bold) (Table 1; Fig. 1 and 2) with an alternate metal-binding ligand negatively impacts metal selectivity and transport rates (26). The functional significance of the remaining three metal-binding sites, B and C1/C2, has not, in general, been examined in detail, but they are thought to play regulatory roles in metal transport. The B site, in particular, has been proposed to stabilize the dimer to allow an alternating access (two-step) model of metal transport (27, 28). A recent small-angle X-ray scattering and molecular dynamics analysis of the structurally characterized CTD of the magnetosome Fe transporter MamM of *Magnetospirillum gryphiswaldense* (29) suggests that Zn binding by the CTD induces a more tightly packed, closed, V-shaped structure like that found in the outward-facing conformation of the full-length *E. coli* YiiP (Fig. 1) (24, 27), relative to a splayed open, apo-state conformation observed in the isolated

CTDs. The findings of previous studies of the CTD from *Thermus thermophilus* CzcB are consistent with those of the studies of the MamM CTD (28, 29). Further *in vivo* characterization of MamM mutants harboring single or double alanine substitutions of putative C1/C2-site ligands found that the mutations disrupted metal transport activity and reduced magnetite crystal formation, suggesting that metal binding to the CTD facilitates metal transport (29).

In addition to *T. thermophilus* CzcB and *M. gryphiswaldense* MamM, functional homologs of *E. coli* YiiP have been identified in a number of microorganisms, including *Shewanella oneidensis* YiiP, *Ralstonia metallidurans* CzcD, *Staphylococcus aureus* CzcB, *Deinococcus radiodurans* MntE, *Rhizobium etli* EmfA, and *Streptococcus pneumoniae* CzcD and MntE (20, 21, 23, 30–35). Previous studies have consistently shown that pneumococcal cells lacking *czcD* are specifically sensitive to Zn toxicity, while *mntE* mutants are sensitive to Mn overload (21, 22, 34, 35). During metal stress, *czcD* and *mntE* mutants accumulate Zn and Mn, respectively. These data suggest that *S. pneumoniae* MntE selectively transports Mn, while CzcD transports Zn. In contrast, *E. coli* YiiP transports at least two metal ions, Zn and cadmium (Cd), with similar efficiencies and likely also transports Fe(II) (23, 36); thus, *E. coli* YiiP is representative of the mixed-metal subclade of the CDFs (see Fig. S1 in the supplemental material). How metal selectivity is achieved for the *S. pneumoniae* CDFs, as well as CDFs in general, remains unclear.

In the present study, we sought to probe the origin of metal selectivity among CDFs. A multiple-sequence alignment of *S. pneumoniae* CzcD, *S. pneumoniae* MntE, and *E. coli* YiiP (Fig. 2) based on a more extensive alignment of 29 CDF superfamily

FIG 2 Multiple-sequence alignment of *Streptococcus pneumoniae* CzcD and MntE with *Escherichia coli* YiiP derived from a more extensive alignment (see Fig. S1 in the supplemental material). Metal-binding residues are highlighted in yellow (site A), blue (site B), dark green (site C1), and light green (site C2). #, the Asp residue that bridges sites C1 and C2; red, residue pairs of the subunit-bridging charge interlock; pink, *S. pneumoniae* CzcD candidate metal-binding residues targeted in this study; gray circles, modestly conserved residues targeted in *S. pneumoniae* MntE (see Fig. S4 in the supplemental material). Secondary structural elements of *E. coli* YiiP are shown as α helices (coils; TM1 to TM6, $\alpha 7$, and $\alpha 8$) and β strands (arrows; $\beta 1$ to $\beta 3$) (24, 53). *E. coli* YiiP and *S. pneumoniae* CzcD and MntE conserved domains are 23 to 27% identical and share $\approx 60\%$ similarity. The residue numbering across the top refers to that for the *E. coli* YiiP sequence. Letters in red font represent the C-terminal residues in YiiP (S300), CzcD (H296), and MntE (E394).

members (see Fig. S1 in the supplemental material) reveals a number of features that may contribute to metal transport specificity in CDFs. First, the B, C1, and C2 metal-binding sites are altered or possibly absent in *S. pneumoniae* CzcD and MntE (Fig. 1). Second, *S. pneumoniae* MntE possesses a long (~ 90 -residue; residues 302 to 394) C-terminal extension, or tail, domain that effectively doubles the size of the *S. pneumoniae* MntE CTD relative to that of the *S. pneumoniae* CzcD and *E. coli* YiiP CTDs (Fig. 2; see also Fig. S1 in the supplemental material). This extension is unrelated to anything present in the protein database but is found in all members of the Mn-specific clade of the CDF superfamily (20). Finally, a phylogenetic comparison of *E. coli* YiiP and mammalian Zn trans-

porters (ZnTs) reveals that the most N-terminal of the four A-site ligands, corresponding to D45 in *E. coli* YiiP, strongly influences metal transport specificity (26). Most members of the Mn-specific clade, including *S. pneumoniae* MntE, in contrast, have an Asn (N47 in MntE) in this position (Fig. 2; see also Fig. S1 in the supplemental material) (20). In this study, we show that metal specificity is dictated largely by the latter feature, in which two of the four A-site residues are primary, although not exclusive, determinants of metal selectivity in CDFs. In total, our functional analysis of CDF paralogs in *S. pneumoniae* improves our understanding of how CDF transporters discriminate among intracellular metal ions to maintain transition metal homeostasis.

TABLE 1 Candidate metal-binding site ligands and charge-interlock pairs in *S. pneumoniae* CzcD and MntE based on the structure of *E. coli* YiiP and targeted for mutagenesis in this work

Metal-binding site	Residue(s) ^a in:		
	<i>E. coli</i> YiiP	<i>S. pneumoniae</i> CzcD	<i>S. pneumoniae</i> MntE
A-site motif (WX-YZ)	DD-HD	HD-HD	ND-DD
A-site ligands	D45	H39	N47
	D49	D43	D51
	H153	H142	D155
	D157	D146	D159
Charge interlock	K77	R71	K79
	D207	D197	D209
B-site ligands	D68	D62	D70
	H71	Y65	H73
	H75	Y69	H77
C1 ligand	H232	N222	<i>K233</i>
	H248	H239	<i>T249^b</i>
C2 ligand	H261	E249	H262
	H283	E270	H284
Bridging (#) ^c C1/C2	D285	D272	E286

^a Residues that play roles in metal transport selectivity are in boldface, while poorly metal coordinating residues of the candidate MntE C1 site are italicized. See Fig. 1 for the structural alignment and Fig. 2 and Fig. S1 in the supplemental material for a multiple-sequence alignment that identifies these residues.

^b Not targeted for mutagenesis in this work.

^c #, candidate metal-coordinating residue that bridges the putative C1 and C2 metal sites (see Fig. 1).

MATERIALS AND METHODS

Reagents. All antibiotics, manganese(II) chloride tetrahydrate, nitrilotriacetic acid (NTA), and anti-FLAG rabbit polyclonal antibody were purchased from Sigma-Aldrich; zinc sulfate was from Alfa Aesar; bovine liver catalase (filtered) was from Worthington Biochemical Corporation; TraceSELECT nitric acid (HNO₃) was from Fluka; and protease inhibitor cocktail III was from Millipore. Bacto brain heart infusion (BHI) broth and BBL Trypticase soy agar with sheep blood were purchased from Becton Dickinson.

Bacterial strain construction. All the strains used in the study were derived from *S. pneumoniae* D39 (also referred to as strain IU1781) and are listed in Table S1 in the supplemental material. All mutant strains were constructed by gene deletion replacement and antibiotic counterselection using the Janus *rpsL*⁺ cassette (37). Briefly, the 0.8- to 1-kb regions upstream and downstream of *czcD* (SPD_1638) and *mntE* (SPD_1384) were amplified from genomic DNA using inner primers containing regions flanking the Janus kanamycin resistance cassette (*kan-rpsL*⁺). The two outside fragments generated were then joined together with the inner *kan-rpsL*⁺ fragment. The final PCR product was transformed into competent *rpsL1* pneumococcal cells using standard techniques. Bacteria were grown on Trypticase soy agar II plates containing 5% (vol/vol) defibrinated sheep blood (TSABII-BA). The plates were incubated at 37°C in an atmosphere of 5% CO₂. For antibiotic selections, TSABII-BA plates contained 250 µg/ml kanamycin or streptomycin.

Site-directed mutagenesis was used to generate amplicons containing allelic substitutions in *czcD* and *mntE*. Briefly, complementary primers were designed so that the mutation of interest was located in the center of the sequence. Overlapping fragments generated by PCR were joined together and transformed into competent IU8449 (Δ *czcD2*) or IU7898

(Δ *mntE2*) cells, and cells were selected for streptomycin resistance. All resulting constructs were confirmed by DNA sequencing.

For construction of C-terminal single-FLAG-tagged CzcD and MntE expressed from their respective native promoters, overlapping 1.5-kb fragments were generated by PCR using primers with the flanking FLAG tag sequence 5'-GATTATAAAGATGATGATGATAAA-3' and the genomic DNA template from the mutant strain of interest. Fragments were then joined together and transformed into competent IU8449 (Δ *czcD2*) or IU7898 (Δ *mntE2*) cells for allelic replacement at the native locus, and cells were selected for antibiotic resistance. All resulting constructs were confirmed by DNA sequencing.

Bacterial growth conditions. BHI medium was of standard composition and prepared with double-distilled water. For growth experiments, bacteria were inoculated into BHI broth from frozen culture stocks and then serially diluted and propagated overnight at 37°C in an atmosphere of 5% CO₂. Exponentially growing cultures that had been grown overnight were diluted to an optical density at 620 nm (OD₆₂₀) of 0.006 in prewarmed BHI medium containing increasing concentrations of ZnSO₄ or MnCl₂. All cell growth at 37°C in an atmosphere of 5% CO₂ was monitored over time.

ICP-MS for total cell-associated zinc or manganese. The total cell-associated Zn or Mn was quantified from 5-ml cultures that had been grown in BHI medium for 2 h with or without 0.2 mM ZnSO₄ or MnCl₂ and analyzed essentially as described previously (34). Briefly, cells were centrifuged, washed once with ice-cold 1× phosphate-buffered saline (PBS), pH 7.4, containing 2 mM NTA, washed twice with ice-cold 1× metal-free PBS (10 g/liter Chelex-100 resin was used to remove the metals), and dried overnight using a centrifuge evaporator. Dried cells were solubilized in 400 µl 30% HNO₃ and lysed by incubation at 95°C for 10 min with shaking at 500 rpm. Samples were prepared for inductively coupled plasma mass spectrometry (ICP-MS) by diluting 300 µl of lysed cells into 2.7 ml 2.5% HNO₃. Metal concentrations were calculated from the standard curve using 1- to 30-ppb metal stock solutions and normalized to the total protein concentration, determined using a DC protein assay (Bio-Rad).

Western blot analysis of CzcD and MntE proteins. Protein expression was determined from strains harboring chromosomal fusions of single-FLAG-tagged CzcD or MntE grown to an OD₆₂₀ of approximately 0.15 in BHI medium and treated with 200 µM ZnSO₄ or 500 µM MnCl₂ for 40 min. Cells were centrifuged; washed with 1× PBS, pH 7.4; resuspended in 1/35 of the original culture volume with 1× PBS, pH 7.4, containing 1% sodium dodecyl sulfate, 0.1% Triton X-100, and 0.5 mg DNase; and lysed at 37°C for 30 min. Cell lysates were diluted into 2× Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol and heated at 95°C for 10 min. Ten micrograms of total proteins was separated by SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% membrane block agent (ECL; GE Healthcare) in 1× PBS, pH 7.4, containing 0.1% Tween 20 (PBST) and probed with anti-FLAG rabbit polyclonal antibody in PBST. Proteins were detected using anti-rabbit immunoglobulin antibody linked to horseradish peroxidase and an enhanced chemiluminescence (ECL) Western blotting reagent. Western blots were imaged using an industrial vision system.

Metal sensitivity assay using a drop test. Exponentially growing cultures that had been grown overnight in BHI medium were allowed to reach an OD₆₂₀ of approximately 0.6 before being serially diluted 10-fold up to a 10⁻⁵ dilution in BHI medium. An aliquot (5 µl) of each dilution was spotted onto BHI agar plates containing 5,000 U of filtered bovine liver catalase and various concentrations of zinc or manganese salts. The plates were photographically documented after 24 h of incubation at 37°C in an atmosphere of 5% CO₂.

RESULTS

The C-terminal extension (tail) of *S. pneumoniae* MntE exhibits a modest role in manganese resistance. Consistent with the find-

FIG 3 *S. pneumoniae* CzcD confers cellular zinc resistance, while MntE confers manganese resistance. Exponentially growing cells were diluted into prewarmed BHI broth containing increasing concentrations of Zn or Mn at time zero and allowed to proliferate. (A, B) Cell density after inoculation and 6 h of growth. Full growth curves are shown in Fig. S2 in the supplemental material. (C, D) Total amount of cell-associated metal measured by ICP-MS. Cells were grown in BHI medium with 200 μ M Zn for *czcD* strains and Mn for *mntE* strains or without added metal. The means for at least three independent cultures $\pm$ SEMs are shown. WT, wild type; Δ tail, strains lacking the C-terminal tail; Δ CTD, strains lacking the CTD.

ings of previous studies, titration of Zn or Mn into a rich growth medium perturbs the growth of pneumococcal cells lacking *czcD* or *mntE*, respectively (Fig. 3A and B; see also Fig. S2 in the supplemental material). Under these growth conditions, ICP-MS data revealed a 2- to 3-fold increase in the total amount of cell-associated Zn and Mn for *czcD*-null and *mntE*-null strains, respectively, compared to that for wild-type cells (Fig. 3C and D). Using this phenotypic growth assay, we first sought to investigate the functional importance of the C-terminal cytoplasmic domain (CTD). Previous studies suggested that the CTD contributes to the CDF function, but its precise role in metal transport remains unclear (18, 25, 38). *S. pneumoniae* strains harboring deletions of the entire CTD in both CzcD (residues 202 to 296) and MntE (residues 215 to 394) just C terminal to the short linker that connects the TMD and the CTD (Fig. 2) gave growth phenotypes like those of null mutants (Fig. 3A and B). This suggests that the N-terminal transmembrane domain of either *S. pneumoniae* CDF cannot function in the absence of the immediately appended CTD.

We next investigated the C-terminal extension, or tail, of *S. pneumoniae* MntE, which we hypothesized may serve an important function since it is present in all CDFs within the Mn-specific clade (see Fig. S1 in the supplemental material). However, to our surprise, removal of the C-terminal tail of *S. pneumoniae* MntE (residues 306 to 394) (Fig. 2) resulted in a phenotype of only modest growth compared to that of the wild-type strain when grown with 200 μ M Mn (doubling times, 55 min for the wild type versus 80 min for the strain without the C-terminal tail) (Fig. 3B; see also Fig. S2E in the supplemental material). Higher Mn concentrations exacerbated the growth phenotype (see Fig. S2F in the supplemental material), and approximately 2-fold higher total cell-associated Mn was observed for the strain without the C-terminal tail than for the wild-type strain (Fig. 3D). These findings

FIG 4 Alterations in the charge interlock or putative metal-binding site B result in zinc sensitivity for *S. pneumoniae* CzcD (A, C) and manganese sensitivity for *S. pneumoniae* MntE (B, D). Exponentially growing cells were diluted into prewarmed BHI broth with (gray) or without (black) metal at time zero and allowed to proliferate. Zn was used at 200 μ M for the experiments with *czcD* strains, while Mn was used at 200 μ M for the experiments with *mntE* strains. The means for at least three independent cultures $\pm$ SEMs are shown. Western blots showing protein expression are shown in Fig. S3 in the supplemental material.

suggest that the C-terminal tail in MntE is required for maximal Mn(II) transport activity.

The charge interlock is important for CDF function. Crystallographic and biochemical studies of *E. coli* YiiP and its close homolog from *S. oneidensis* (see Fig. S1 in the supplemental material) have revealed that the conserved residue quartet K77 and K77' (from the opposite protomer) in the TM2-TM3 loop and D207 and D207' just C terminal to TM6 forms a salt-bridging network that stabilizes the dimer and may help to coordinate a conformation change within the transmembrane domain upon zinc binding to the CTD (25, 27) (Fig. 1). Single charge-reversal substitutions of these residues in YiiP (K77D or D207K) were shown to reduce protein stability and negatively impact metal transport rates *in vitro*, while the double-charge-reversal mutant (K77D/D207K) appeared to regain function (25). Our alignment of the sequences of *S. pneumoniae* CzcD and *S. pneumoniae* MntE with the sequence of *E. coli* YiiP predicted that the pneumococcal CDFs also harbor a charge-interlock pair (R71 [or K70]/D197 in CzcD and K79/D209 in MntE) (Fig. 2). Indeed, we observed that reversing the charge of either residue in either pneumococcal CDF (R71D and D197K in CzcD, K79D and D209K in MntE) resulted in a growth phenotype similar to that of null mutants when cells were grown with excess metal (Fig. 4A and B). Interestingly, the growth defect observed in the single mutants was not restored in strains harboring the compensatory, double-charge-reversal mutant, CzcD K70D/D197K or R71D/D197K or MntE K79D/D209K (Fig. 4A and B), even though all proteins were expressed at a level exceeding the level of wild-type CzcD expression in cells (see Fig.

S3 in the supplemental material). Our data do not address the possibility of altered protein stability over time, as this was not examined. However, we note that the charge polarity of the charge interlock (N terminus, basic; C terminus, acidic) appears to be invariant among all CDFs (see Fig. S1 in the supplemental material), and thus, a charge reversal may not be generally tolerated, perhaps as a result of recruitment of one of the acidic residues into the B-site coordination sphere (see below; Fig. 1) or an inability to access an active, dimeric conformation.

Site B is important for CzcD and MntE function. The Zn(II) ion bound to site B of the *E. coli* YiiP structure is proximal to the charge interlock and adopts a tetrahedral coordination geometry with one Asp residue (D68 in *E. coli* YiiP), two His residues (H71 and H75), and a water molecule as ligands (Fig. 1; Table 1) (24, 25). This site is not highly conserved within the CDF superfamily (see Fig. S1 in the supplemental material), and alignment of the *S. pneumoniae* CzcD sequence with the *E. coli* YiiP sequence reveals that site B may be altered or absent in *S. pneumoniae* CzcD, since only the N-terminal-most Asp is retained, with the two His residues being replaced by Tyr (Fig. 1 and 2). If it is isostructural with *E. coli* YiiP, this would create an Asp-Tyr₂ site, which would appear to be better suited to bind Fe(III) than Zn(II); Tyr residues are, however, present in $\approx 0.1\%$ of all functionally characterized Zn(II) metalloprotein sites (39–42). Mutational analysis of site B in *S. pneumoniae* CzcD (D62, Y65, and Y69) revealed that strains expressing single-amino-acid-substitution mutants CzcD Y65A and CzcD Y69A gave growth phenotypes similar to the phenotype observed for the *czcD*-null mutant during Zn toxicity (Fig. 4C). Since site B is located near the charge interlock (Fig. 1), mutations made within this region may alter the transporter conformation or lead to protein instability; it is also not known if the Tyr residues conserved in this region function directly as Zn(II) ligands. We noted robust cellular expression of the CzcD Y65A and Y69A B-site mutants, but no expression was observed for the D62A mutant (see Fig. S3A in the supplemental material). Future studies designed to probe the effect of the replacement of Y65 and Y69 with Phe or Trp, instead of Ala, which is found naturally in other members of the Zn-specific CDF clade (see Fig. S1 in the supplemental material), will provide direct insights into B-site metal coordination.

In contrast to *S. pneumoniae* CzcD, all three site B ligands of *E. coli* YiiP were conserved in *S. pneumoniae* MntE (D70, H73, and H77) (Fig. 2). Pneumococcal strains expressing the D70A, H73A, or H77A variant of *S. pneumoniae* MntE were as sensitive to Mn toxicity as the $\Delta mntE$ strain (Fig. 4D). Like the analogous substitutions in CzcD, MntE H73A and H77A mutant proteins were expressed to wild-type levels in cells, with no expression being observed for the MntE D70A mutant (see Fig. S3B in the supplemental material). These findings suggest that *S. pneumoniae* MntE likely harbors the metal-binding site B, although the nature of the metal bound here cannot be determined from these experiments.

Sites C1 and C2 are dispensable for MntE function, in contrast to their dispensability in CzcD. Sites C1 and C2 in *E. coli* YiiP form a subunit-bridging binuclear cluster that is located at the base of the V-shaped dimeric CTD in the closed conformation (Fig. 1). The two symmetry-related clusters of the dimer (4 Zn ions per dimer) are solvent exposed and separated by ≈ 16.5 Å (25, 27). Both C1 and C2 metal ions are similarly coordinated by two terminal histidines (H232 and H248 in C1, H261' from the opposite protomer and H283 in C2) and a bidentate Asp (D285) func-

tioning as a bridging ligand to both C1 and C2 metal ions (Fig. 1; Table 1). The fourth ligand to the C1 and C2 Zn(II) ions is likely a solvent molecule but is not resolved in these structures. This coordination structure is analogous to that found in the structure of the isolated CTD dimer from a thermophilic CzcB, with Zn(II) binding being known to drive a structural change in the dimer (28). Filling of the C1 site may be communicated directly to the A site, since the reactivity of C157 in a D157C A-site mutant in *E. coli* YiiP (DD-HD to DD-HC, where the mutation residues are in bold) (Table 1) toward an exogenous alkylating agent (*N*-ethylmaleimide) was perturbed in an H232A (C1-site) mutant but not in wild-type YiiP (25). These findings suggest that metal binding to the C1/C2 sites triggers a conformational change in the transport region that may be required for transporter cycling between inward- and outward-facing conformations (25). An *in vitro* analysis of *E. coli* YiiP H232A showed that the K_m for Zn(II) and the turnover rate were modestly affected relative to those in wild-type YiiP, providing support for functionally important cross talk between the two domains (25). We therefore sought to identify the residues within the cytoplasmic CTD in CzcD and MntE in *S. pneumoniae* that may contribute to cellular metal resistance.

A multiple-sequence alignment of the *S. pneumoniae* CzcD and MntE sequences with the *E. coli* YiiP sequence suggests that sites C1/C2 may be altered or missing altogether from both *S. pneumoniae* CzcD and MntE (Fig. 2; Table 1), since neither protein sequence contains the complete HX₁₆HX₁₃HX₂₂HXD binding motif for the site C1/C2 cluster in *E. coli* YiiP. Site C1 is the most variable between the two transporters, appears to be completely absent in *S. pneumoniae* MntE (K233 and T249) (Table 1), and is altered in *S. pneumoniae* CzcD (N222, corresponding to H232 in YiiP, and H239) (Table 1). In the C2 site of CzcD, both His residues align with Glu residues (E249 and E270), while a bridging Asp (D272) is retained (Table 1). In MntE, the C2 site is retained as E262 and H284, with E286 being a candidate bridging carboxylate (Fig. 1 and 2; Table 1).

Single amino acid substitutions of the putative C1-site ligand (H239A) as well as the putative bridging carboxylate (D272A) in *S. pneumoniae* CzcD led to cellular Zn sensitivity indistinguishable from that of a *czcD*-null strain (Fig. 5A), despite being expressed to high levels in cells (see Fig. S3A in the supplemental material). In contrast, the strain with the C1-site N222A mutation retained nearly wild-type-like resistance to Zn toxicity (Fig. 5A). With respect to the C2-site ligands in *S. pneumoniae* CzcD, changing E249 to a non-metal-binding Ala resulted in only a slight decrease in Zn resistance that was comparable to that of the C1-site mutant (N222A), while an E270A substitution abolished Zn resistance relative to that of a wild-type strain (Fig. 5A). It is interesting to note that N222 (C1 site) and E249' (C2 site) would represent the most distal terminal ligands of a YiiP-like binuclear cluster (Fig. 1), with the remaining functionally required residues (H239, E270, and D272, corresponding to H248, H283, and D285 in YiiP, respectively) being ideally positioned to coordinate only a single Zn ion in a YiiP-like site. These data suggest that *S. pneumoniae* CzcD does indeed require Zn binding to the C-site region of the CTD to effect zinc resistance and the transporter function in cells; however, the stoichiometry and metal-binding site structure are not defined by these data.

In contrast to its dispensability in *S. pneumoniae* CzcD, the C2 metal-binding site, although conserved in *S. pneumoniae* MntE (E262, H284, and E286) (Table 1), is largely dispensable for cellu-

FIG 5 Sites C1 and C2 are dispensable for *S. pneumoniae* MntE function but not for CzcD function in cells. Exponentially growing cells were diluted into prewarmed BHI broth with (gray) or without (black) metal at time zero and allowed to proliferate. Zn was used at 200 μ M for the experiments with *czcD* strains (A), while Mn was used 200 μ M for the experiments with *mntE* strains (B). Putative metal-binding sites C1 and C2 and the bridging ligand (#) are shown. The means for at least three independent cultures $\pm$ SEMs are shown. Western blots showing protein expression are shown in Fig. S3 in the supplemental material.

lar Mn resistance since the ability of MntE to confer cellular Mn resistance was not eliminated in strains harboring Ala substitutions of these residues (H262A, H284A, and D286A) (Fig. 1 and 2) individually or in combination (Fig. 5B). In an effort to identify other residues that may contribute to CzcD or MntE function, we systematically replaced other candidate metal-binding residues (Glu, Asp, His, and Asn) in the C1/C2 metal-binding-site region of each CTD with Ala. To our surprise, no impact on cellular Mn resistance was seen in any of the *S. pneumoniae* MntE variants tested, including those with single and double Ala substitutions of 10 bacterially conserved residues (N243, N247, N253, E260, E263, E269, D280, D282, E292, or E294), and the strains had growth yields similar to those of wild-type cells (see Fig. S4 in the supplemental material). These data taken collectively suggest that YiiP-like C1/C2 sites are missing entirely or that they contribute minimally to the function of *S. pneumoniae* MntE, in contrast to their role in CzcD. Further biophysical studies are under way to establish if C1/C2 metal-binding sites are indeed present in *S. pneumoniae* MntE.

Site-A residues enforce metal specificity in MntE and CzcD. Hoch and coworkers performed a phylogenetic comparison between *E. coli* YiiP and mammalian Zn transporter (ZnT) homologs (26). They noted that the N-terminal-most A-site residue differed between the two, with D45 of *E. coli* YiiP in the DD-HD (D45–D49–D153–D157) motif being a His in the mammalian ZnTs (HD-HD) (Table 1; see also Fig. S1 in the supplemental material). They demonstrated that the conversion of D45 to a His

FIG 6 Site-A ligands in *S. pneumoniae* CzcD are required for cellular zinc resistance. (A) Mutations within the transmembrane metal transport A site abolish cellular Zn resistance. Exponentially growing cells were diluted into prewarmed BHI broth with (gray) or without (black) 200 μ M Zn at time zero and allowed to proliferate. The means for at least three independent cultures $\pm$ SEMs are shown. Western blots showing protein expression are shown in Fig. S3 in the supplemental material. (B) Drop test analysis of the wild-type strain versus several *czcD* variants whose functions mimic that of an Mn transporter on BHI agar supplemented with increasing concentrations of Mn. All *czcD* mutants lacked the Mn exporter MntE in order to demonstrate Mn transport by the CzcD variants. Cells were grown to an OD₆₂₀ of approximately 0.6, serially diluted, and then spotted onto plates, starting from the undiluted sample (top) down to the 10^{−5} dilution (bottom).

to create a mammalian ZnT-like HD-HD motif in YiiP significantly reduced cadmium transport by *E. coli* YiiP, thus making the transporter more selective for Zn. Likewise, exchanging the His to an Asp in the mammalian ZnTs to create an *E. coli* YiiP-like transporter DD-HD motif resulted in increased promiscuity in metal transport.

A multiple-sequence alignment showed that *S. pneumoniae* CzcD is similar to mammalian ZnTs in that it also differs from *E. coli* YiiP by a single residue, H39 versus D45 (HD-HD; H39–D43–H142–D146) (Fig. 2). In contrast, the A site in *S. pneumoniae* MntE differs by two residues, with D45 and H153 of the *E. coli* YiiP DD-HD motif being an Asn and an Asp in *S. pneumoniae* MntE, respectively (ND-DD; N47–D51–D155–D159) (Fig. 2; Table 1). We first performed a mutational analysis in which each putative metal-binding ligand in the A site was independently changed to Ala for both *S. pneumoniae* CzcD and MntE. Zn and Mn resistance was abolished in all Ala variants of *S. pneumoniae* CzcD and MntE, respectively, resulting in a strong reduction in growth yield (Fig. 6A and 7A), even though the proteins were expressed in the mutant cells at or above the levels at which they were expressed in wild-type cells (see Fig. S3 in the supplemental material). Next, we converted the A-site residues of *S. pneumoniae* CzcD so that the A-site sequence resembled that of a MntE or Mn-clade transporter (see Fig. S1 in the supplemental material). As expected, pneumococcal cells expressing *czcD* H39N or *czcD* H142D alleles independently or in combination consistently showed Zn sensitivity (Fig. 6A), even though these mutant proteins were expressed at levels above the levels at which they were expressed in wild-type cells

FIG 7 Site-A N47 and D155 ligands of the ND-DD motif are required for the *S. pneumoniae* MntE function. (A) Mutations within the transmembrane metal transport A site abolish cellular Mn resistance. Exponentially growing cells were diluted into prewarmed BHI broth with (gray) or without (black) 200 μ M Mn at time zero and allowed to proliferate. The means for at least three independent cultures $\pm$ SEMs are shown. Western blots showing protein expression are shown in Fig. S3 in the supplemental material. (B) Drop test analysis of the wild-type strain versus *mntE* variants whose functions mimic that of a Zn transporter on BHI agar supplemented with increasing concentrations of Zn. All *mntE* mutants lack the Zn exporter CzcD in order to demonstrate Zn transport by the MntE variants. Cells were grown to an OD₆₂₀ of approximately 0.6, serially diluted, and then spotted onto plates, starting from the undiluted sample (top) down to the 10⁻⁵ dilution (bottom).

(see Fig. S3A in the supplemental material). This reveals that these MntE-CzcD functional chimeras are unable to transport Zn. The consistently increased ($\approx$ 10-fold) expression of all nonfunctional CzcD transporters is likely a direct result of the increased cellular accumulation of Zn and a corresponding increase in the SczA-dependent activation of *czcD* allele expression (see Fig. S3A in the supplemental material). We next transferred these CzcD MntE-like A-site mutants (N39N, H142D, and H39N/H142D) into an *mntE*-null parent strain to determine if any of the mutants could relieve the Mn sensitivity. By the drop test, we observed that only

the *S. pneumoniae* CzcD H39N/H142D double mutant variant could grow in the presence of higher concentrations of Mn than the $\Delta mntE$ strain but not to the full extent of a wild-type *mntE* strain (Fig. 6B; see also Fig. S5 in the supplemental material). Complete rescue may not have been achieved because the residual Zn concentration in the BHI growth medium (20 μ M) was not high enough to fully activate the Zn-responsive regulator SczA (35) to allow robust CzcD expression (see Fig. S3A in the supplemental material).

In a reciprocal experiment, we exchanged the A-site residues in *S. pneumoniae* MntE (ND-DD motif) to resemble those of a Zn-like transporter, with one construct having a sequence mimicking the metal-binding motif for *E. coli* YiiP (DD-HD motif) and another having a sequence resembling that of *S. pneumoniae* CzcD (HD-HD motif). Pneumococcal cells harboring *S. pneumoniae* MntE alleles N47D, N47H, D155H, N47D/D155H, and N47H/D155H showed diminished growth when they were challenged with Mn (Fig. 7A). Interestingly, when placed in a *czcD*-null mutant background to survey possible Zn transport by these YiiP- and CzcD-like *S. pneumoniae* MntE mutants, some protection against Zn toxicity was seen in all but one strain, the MntE N47D strain, but again, the protection was not to the extent observed for the strain with wild-type *czcD* (Fig. 7B). Although the MntE N47D mutant was expressed at wild-type levels (see Fig. S3B in the supplemental material), the four candidate Asp ligands in the first coordination shell are expected to electrostatically destabilize the A-site region as a result of charge imbalance. It is interesting to note, however, that the YiiP- and CzcD-like double mutations, the MntE N47D/D155H and MntE N47H/D155H mutations, provided significant protection against Zn toxicity in a $\Delta czcD$ strain background. Unlike *S. pneumoniae* CzcD, *S. pneumoniae* MntE appeared to be constitutively expressed, as protein expression was not affected by either Mn stress, Zn stress, or metal deprivation induced by addition of EDTA to the cells (see Fig. S3B in the supplemental material). Since full rescue was not achieved in any A-site functional chimeric transporter in the corresponding null mutant strain background, other determinants beyond the A-site TMD must impact metal selectivity and/or metal transport rates.

DISCUSSION

This work provides the first extensive *in vivo* functional analysis of two paralogous CDF family transporters, *S. pneumoniae* CzcD and MntE, which must efficiently function in a common cytoplasm. We conclude here that the ability of a CDF to discriminate between Zn(II) and Mn(II) is largely dictated by the nature of the first (W) and third (Y) residues within a WX-YZ motif (Table 1), where W and Y correspond to opposite corners of the A-site metal complex (D45, H153 in YiiP) (Fig. 1). We show here that W and Y must be Asn and Asp, respectively, in the Mn-specific CDF clade (ND-DD) and two His residues in the Zn-specific clade (HD-HD), in order to achieve specific cellular resistance to Mn(II) and Zn(II) toxicity, respectively (Fig. 6 and 7). The discrimination between these metal ions may have been accomplished by mutational drift in the evolution of the transport site (site A), similar to what was previously discussed for mammalian ZnTs and *E. coli* YiiP (26). These differences in A-site metal coordination chemistry are reinforced by the clear functional distinctions that we have mapped to the cytoplasmic CTDs of each transporter largely as a consequence of anticipated distinct C-site metal-binding characteristics (Fig. 5). The CTD of CzcD clearly harbors at least one

Zn-binding site that is essential for cellular Zn resistance. In contrast, using the same targeted mutagenesis strategy, coupled with the single and multiple substitution of 10 additional conserved, potential metal-binding residues (see Fig. S4 in the supplemental material), we were unable to identify a strong candidate metal-binding site in the CTD of MntE required for cellular Mn resistance.

Our findings suggest that the nature of the first coordination shell of A-site ligands is a primary specificity determinant that enhances the transport of cognate versus noncognate or competing metal ions. Coordination number and geometry are the likely discriminating factors, since Mn often adopts five- or six-coordinate trigonal bipyramidal or octahedral geometries, while Zn strongly favors four-coordinate tetrahedral or distorted tetrahedral geometries (42). *S. pneumoniae* MntE may exploit this preference in coordination chemistry to select for Mn over Zn and other transition metals by employing the metal-binding motif pair N47-X₃-D51 and D155-X₃-D159 on transmembrane helices TM2 and TM5, respectively, where N47 and D155 play significant roles in metal ion selection (Fig. 6 and 7). Bidentate coordination by N47 or D155 could give rise to a five-coordinate complex in MntE, thus enforcing Mn(II) specificity. Consistent with this, simply changing these two residues to CzcD-like His residues results in an *S. pneumoniae* MntE capable of providing some degree of protection against Zn toxicity (Fig. 7B). The reciprocal is also true, in that CzcD harboring an MntE-like A site provides significant protection against Mn toxicity in a $\Delta mntE$ strain (Fig. 6B).

A recent study on the bacterial natural resistance-associated macrophage protein (NRAMP) DMT from *Staphylococcus capitis* reveals an Asn in the first coordination sphere in a trigonal bipyramidal Mn-binding site required for metal transport (43). The coordinating Asn was reported to be bidentate, although both metal coordination bonds were very long (2.9 and 3.5 Å), with the carboxamide oxygen atom making a closer approach to the metal (43). An Asn in the first coordination shell is also found in formiminoglutamase, an arginase-like di-Mn(II) metalloenzyme, from *Trypanosoma cruzi* (44), and replacement of this Asn with His resulted in marginally lower catalytic activity. In authentic di-Mn arginases, a His residue occupies this position (44). In contrast, Asn is unlikely to be required for metal selection or transport in Mn-specific P-type ATPases, e.g., the cation transporter protein C (CtpC) from *Mycobacterium tuberculosis* (45), or in the Mn-specific ATP-binding cassette (ABC) uptake transporter PsaBCA from *S. pneumoniae*. In the latter case, metal discrimination is dictated by monodentate versus bidentate coordination by a single carboxylate ligand in the solute-binding component of the transporter PsaA, giving rise to distinct dissociation rates for cognate (Mn) versus noncognate (Zn) metals (45, 46). It will be interesting to determine how widespread the use of Asn is to enhance Mn specificity among functionally and structurally diverse classes of bacterial Mn transporters.

Our findings reveal that the functional features previously ascribed to sites B and C1/C2 present in the CTD of CDFs on the basis of *E. coli* YiiP may not be generally conserved for all CDFs. Structural studies reveal that site B is located in close proximity to the charge-interlock quartet (25, 27) (Fig. 1) and thus may play an important role in CDF function. Our data are consistent with the conservation of the B-site function in both CzcD and MntE, but details of the coordination and identity of the metal bound here will have to await further studies. As for the C1/C2 binuclear clus-

ter, the leading hypothesis remains that the CTD is involved in some way in regulation of metal transport (25), since the N-terminal transmembrane domains of MntE and CzcD fail to function in the absence of an appended CTD (Fig. 3). Similar observations have been made for MamM in *M. gryphiswaldense*, in which C-terminal truncated MamM variants fail to form magnetite (38). However, as highlighted above, our functional data, coupled with a multiple-sequence alignment, provide no support for the existence of a C-site cluster in MntE. We propose that an alternate regulatory mechanism, perhaps in collaboration with the unique C-terminal tail, might be used to stimulate metal translocation through MntE. The structure and biochemical function of the tail are unknown, but it is interesting to note that the ≈ 20 C-terminal residues are rich in basic amino acids, suggesting that they play a role in electrostatic stabilization of the MntE dimer and/or activation of metal transport, as previously described for the *Corynebacterium glutamicum* betaine transporter BetP (47, 48). An alternative possibility not tested here is that the CTD physically interacts with an as yet unidentified accessory protein or metal chaperone to influence metal transport activity, as previously described for MamB (38). This is not unprecedented, as other metal transporters have been shown to employ similar mechanisms to regulate their activities (49, 50). For example, the *E. coli* Mn transporter MntP may be inhibited by expression of the small protein MntS (8). Additional biochemical and structural studies are under way to ascertain the role and the importance of the *S. pneumoniae* MntE tail region and the putative metal-binding sites located within the CTD.

Characterization of a novel subfamily of CDFs lacking the CTD altogether challenges our basic understanding of the role of the CTD in the activation of metal transport (18). CTD-lacking CDFs possess the entire TMD (TM1 to TM6) and appear to conserve the charge-interlock quartet, which is followed by ≈ 15 additional residues, i.e., residues that go into the $\alpha 7$ helix of classical CDFs (see Fig. S1 in the supplemental material) (51). These transporters obviously retain the TMD A site but lack metal-binding sites B and C1/C2 (18) (see Fig. S1 in the supplemental material). Interestingly, the third residue in the *E. coli* YiiP A-site motif DD-HD, defined here to be one of the major metal specificity determinants, is replaced with a Cys in all CTD-lacking CDFs (see Fig. S1 in the supplemental material). There are also several conserved Cys residues positioned in the extreme N terminus relative to the sequences of classical CDFs like *E. coli* YiiP. These changes may have implications in metal selectivity, similar to what we have demonstrated here. In fact, the representative CTD-lacking family member MmCDF3 from *Maricaulis maris* MCS10 is capable of binding Zn and cadmium but not Fe or Co (52). Overexpression of MmCDF3 in cells of an *E. coli* Zn-sensitive strain ($\Delta zitB \Delta zntA$) increased Zn resistance by about 2-fold compared to that for cells containing the empty vector (52). These findings reveal that CTD-lacking CDFs are capable of transporting metal without the involvement of an appended CTD, but how metal specificity is established without it is unclear. Additional studies are required to establish the role for which these CTD-lacking CDFs are utilized in host organisms and whether they interact with an as yet unidentified independently expressed CTD-like protein.

In summary, our findings extend our understanding of how the ubiquitous class of classical CDF transporters discriminates among transition metals. This is an important aspect of cellular metallostasis, as bacteria must continually adapt to changes in

environmental metal availability and in establishing prolonged infection in eukaryotic host organisms. Our findings lay the groundwork for more detailed biochemical and structural studies of the mechanisms of metal transport by CzcD and MntE.

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